Establishment of *in vitro*-infection models for *Chlamydia trachomatis* based on human primary cells and primary tissue

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von

Dipl.-Biol. Julia Zielecki, geb. Schöning
aus Marburg

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Jan-Hendrik Olbertz

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I
Prof. Dr. Andreas Herrmann

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1. Gutachter: Prof. Dr. Thomas F. Meyer
2. Gutachter: Prof. Dr. Richard Lucius
3. Gutachter: Prof. Dr. Stefan Bereswill

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Abstract

Zellkultursysteme mit Krebszelllinien werden seit Langem zur Untersuchung der Interaktion zwischen Pathogenen und ihren Wirtszellen eingesetzt. Diese Systeme eignen sich aufgrund der reduzierten Komplexität für die Analyse einzelner Faktoren, spiegeln jedoch nicht den Zustand primärer Zellen oder die komplexe Gewebestruktur wieder. Um die Beschränkungen zu umgehen, wurden in dieser Arbeit neue Modelle etabliert auf der Grundlage von reversibel immortalisierten humanen Primärzellen und *ex vivo* Kultur von intaktem humanem Eileitergewebe. Infektionen mit dem humanpathogenen Bakterium *Chlamydia trachomatis*, welches chronische Schmerzen oder Unfruchtbarkeit auslösen kann, wurden in diesen Modellen untersucht. Reversible Immortalisierung wurde mit primären human Eileiterzellen (FT Zellen) und humanen Nabelschnurzellen (HUVEC) durchgeführt. Das System basiert auf lentiviralem Gentransfer und dem Cre-lox-System. HUVEC Zellen wurden mit Kombinationen der Onkoproteine hTERT, SV40T und Bmi1 immortalisiert. Immortalisierung von FT Zellen wurde mit SV40T und Bmi1 erreicht. Eine Analyse der FT Zelllinien zeigte Veränderungen des Karyotyps durch die Immortalisierung. Bemerkenswerterweise konnten die Stammzellmarker CD44 und Oct4 in FT Zellen nachgewiesen werden. *Ex vivo* Gewebekultur humaner Eileiter wurde als stabiles Infektionsmodel für *Chlamydia trachomatis* etabliert. Mittels hochauflösender Konfokalmikroskopie wurde gezeigt, dass die Infektion mit *C. trachomatis* tiefgreifende Veränderungen im Epithel der Mukosa auslöst und zum Verlust der Zelladhäsion und Zellpolarität führt. Ein erhöhter Anteil apoptotischer Zellen wurde nach Infektion mit Serovar D beobachtet, einem klinischen Isolat des Genitaltraktes. Dieses Ergebnis steht im Gegensatz zu Infektionen mit dem Laborstamm Serovar L2. Phänotypische Veränderungen in nicht infizierten Zellen weisen auf die Existenz parakriner Signalwege während der akuten Infektion und Veränderung der epithelialen Homeostase hin.

Primärzellen

Reversible Immortalisierung

Eileiter

Chlamydia trachomatis

Abstract

Cell culture systems with cancer-derived cell lines have long been used to study the interaction between pathogens and their host cells. Due to reduced complexity these systems are convenient for the analysis of single factors; however, they do not represent the condition of primary cells or the complex tissue structure. To circumvent these limitations new models were established in this study on the basis of reversibly immortalized human primary cells and *ex vivo* culture of intact human fallopian tube tissue. Infections with the human pathogenic bacterium *Chlamydia trachomatis*, which can lead to chronic pain or infertility, were analyzed in these models. Reversible immortalization was applied to primary human fallopian tube (FT) cells and human umbilical vein cells (HUVEC). This system is based on lentiviral gene transfer and the Cre-lox-system. HUVEC cells were immortalized with a combination of two of the oncoproteins hTERT, SV40T and Bmi1. Immortalization of FT cells was achieved with SV40T and Bmi1. Analysis of FT cell lines revealed changes of the karyotype induced by immortalization. Remarkably, the stem cell markers CD44 and Oct4 were detected in FT cells. *Ex vivo* tissue culture of human fallopian tubes was established as stable and reliable infection model for *Chlamydia trachomatis*. Via high resolution confocal analysis the infection with *C. trachomatis* was discovered to trigger profound changes in the epithelial mucosa, causing loss of cell adhesion and polarity. Interestingly, an increase in the rate of apoptotic cells was observed after infection with serovar D, a clinical genital tract isolate. This finding is in contrast to infections with serovar L2, a laboratory strain. Phenotypic changes in non-infected cells suggest the existence of paracrine signalling during acute infection and change in epithelial homeostasis.

primary cells

reversible immortalization

fallopian tube

Chlamydia trachomatis

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Zusammenfassung

Zellkultursysteme werden seit Langem zur Untersuchung der Interaktion zwischen Pathogenen und ihren lebenden Wirtszellen eingesetzt. Krebszelllinien und immortalisierte Zelllinien sind aufgrund der reduzierten Komplexität der Systeme geeignet für die Analyse einzelner Faktoren. Diese Zellen spiegeln jedoch nicht den Zustand primärer Zellen wieder, und die direkte und routinierte Arbeit mit Primärzellen ist oft kompliziert aufgrund der limitierten Proliferation und Verfügbarkeit der Zellen. Des Weiteren unterscheiden sich diese Modelsysteme von der Infektionssituation *in vivo*, in der mehrere Zelltypen interagieren und die Zellen in die komplexe Gewebestruktur eingebettet sind.

Daher werden neue Modelsysteme auf der Grundlage von Primärgewebe benötigt, um diese komplexe Situation besser nachzustellen. Um die Beschränkungen der herkömmlich verwendeten Zellkulturen zu umgehen, wurden in dieser Arbeit neue Modelle etabliert auf der Grundlage von 1. isolierten reversibel immortalisierten humanen Primärzellen, und 2. *ex vivo* Kultur von intaktem humanem Eileitergewebe. Infektionen mit dem humanpathogenen Bakterium *Chlamydia trachomatis*, welches chronische Schmerzen oder Unfruchtbarkeit auslösen kann, wurden in diesen Modellen untersucht.

Reversible Immortalisierung, d.h. Immortalisierung von Zellen mit der Möglichkeit, die Immortalisierungskonstrukte später aus dem Genom herauszuschneiden, wurde mit primären human Eileiterzellen (FT Zellen) und humanen Nabelschnurzellen (HUVEC) durchgeführt. Das System basiert auf lentiviralem Gentransfer und dem Cre-lox-System. Die HUVEC Zellen wurden mit einer Kombination aus zwei der Onkoproteine hTERT, SV40T und Bmi1 oder nur SV40T immortalisiert. Insgesamt wurden 21 FT Zelllinien hergestellt, welche die Onkoproteine SV40T und Bmi1 enthielten. Eine Analyse der FT Zelllinien zeigte Veränderungen des Karyotyps durch die Immortalisierung auf. Bemerkenswerterweise konnten die Stammzell- und mesenchymalen Marker CD44, Oct4 und N-Cadherin in FT Zellen nachgewiesen werden.

In dieser Arbeit konnte die *ex vivo* Gewebekultur humaner Eileiter erfolgreich etabliert werden und erwies sich als stabiles und verlässliches Infektionsmodel für *Chlamydia trachomatis*. Dies ist die erste Studie, die eine detaillierte Analyse subzellulärer Marker während des Verlaufs der Chlamydieninfektion direkt in humanem Gewebe mittels Immunfluoreszenz beschreibt.

Unter Verwendung hochauflösender Konfokalmikroskopie fanden wir heraus, dass die Infektion mit *C. trachomatis* tiefgreifende Veränderungen im Epithel der Mukosa auslöst und zu einem Verlust der Zelladhäsion und Zellpolarität führt. Interessanterweise wurde außerdem ein Anstieg des Anteils apoptotischer Zellen nach Infektion mit dem Serovar D beobachtet. Das Serovar D ist weit verbreitet unter klinischen Isolaten des Genitaltraktes.

Dieses Ergebnis steht im Gegensatz zu Infektionen mit dem Serovar L2, einem weit verbreiteten Laborstamm. Phänotypische Veränderungen in nicht infizierten Zellen könnten bedeutend für die durch Chlamydien verursachten Symptome sein und zum Verständnis dieser beitragen, da sie auf die Existenz parakriner Signalwege während der akuten Infektion und Veränderung der epithelialen Homeostase hinweisen.

Summary

Cell culture systems have long been used to study the interaction between pathogens and their living host cells. Cancer-derived cell lines and immortalized cells are convenient for the analysis of single factors due to reduced complexity of the systems. These cells, however, do not represent the condition of primary cells, and the direct and routine work with primary cells often is complicated due to the cells' limited proliferation and acquisition. In addition, all of these model systems differ from the infection situation *in vivo*, where multiple cell types interact and the cells are embedded in the complex tissue structure.

Therefore, new primary tissue based model systems are needed to better mimic this complex situation. To circumvent the limitations of traditionally used cell cultures, new models were established in this study on the basis of (1) isolated reversibly immortalized human primary cells, and (2) *ex vivo* culture of intact human fallopian tube tissue. Infections with the human pathogenic bacterium *Chlamydia trachomatis*, which can lead to chronic pain or infertility, were analyzed in these models.

Reversible immortalization, i.e. immortalization of cells with the possibility to excise the immortalizing constructs later from the genome, was applied to primary human fallopian tube (FT) cells and human umbilical vein cells (HUVEC). This system is based on lentiviral gene transfer and the Cre-lox-system. HUVEC cells were immortalized with a combination of two of the oncoproteins hTERT, SV40T and Bmi1 or SV40T alone. In total 21 FT cell lines were generated containing SV40T and Bmi1 oncoproteins. Analysis of FT cell lines revealed changes of the karyotype induced by immortalization. Remarkably, the stem cell and mesenchymal markers CD44, Oct4 and N-cadherin were detected in FT cells.

Ex vivo tissue culture of human fallopian tubes was successfully established in this study and shown to be a stable and reliable infection model for *Chlamydia trachomatis*. This is the first study presenting detailed analysis of subcellular markers by immunofluorescence during the course of chlamydial infection directly in human tissue.

By using high resolution confocal analysis we discovered that infection with *C. trachomatis* triggers profound changes in the epithelial mucosa, causing loss of cell adhesion and polarity. Interestingly, an increase in the rate of apoptotic cells was observed, too, after infection with serovar D, which is prevalent among clinical genital tract isolates. This finding is in contrast to infections with serovar L2, a widely used laboratory strain. Phenotypic changes in non-infected cells could be significant for and contribute to the understanding of *Chlamydia*-induced pathology, as they suggest the

existence of a paracrine signalling during acute infection and change in epithelial homeostasis.

1 Introduction

1.1 Chlamydiae

Chlamydiae are non-motile Gram-negative bacteria. In the past, chlamydiae were thought to be protozoans due to the presence in a typical intracellular vacuole. As the pathogens are obligate intracellular and small (approximately 300 nm for the infectious form), they were later regarded as large viruses. In the 1960's, chlamydiae were finally identified as bacteria (Schachter and Caldwell, 1980; Moulder, 1966; Gear,, et al., 1963). Chlamydiae are distributed world-wide and infect humans and animals (see chapter 1.2).

1.1.1 Taxonomy of chlamydiae

The taxonomy of the order *Chlamydiales* was redefined approximately 10 years ago based on phylogenetic analysis of the 16S and 23S rRNA genes in addition to genetic and phenotypic information (Bush and Everett, 2001; Horn,, et al., 2000; Everett, et al., 1999). The order now contains four families: *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae*. The formerly single genus *Chlamydia* of the family *Chlamydiaceae* was divided into the two genera *Chlamydia* and *Chlamydophila* (Fig. 1.1). However, the proposal was not universally accepted (Stephens, et al., 2009).

Among the *Chlamydiaceae* are human pathogens as well as animal-infecting species. *Chlamydia trachomatis* and *Chlamydophila pneumoniae* are human pathogens causing ocular and urogenital diseases as well as pneumonia (see below). In contrast to members of the *Chlamydiaceae*, bacteria of the families *Parachlamydiaceae* and *Simkaniaceae*

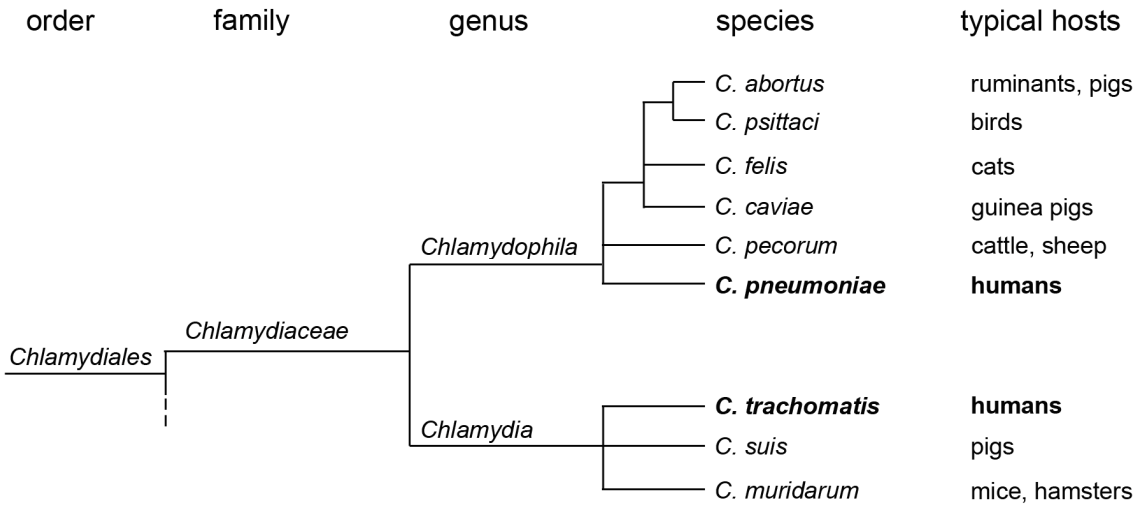


Fig. 1.1: Taxonomy of the family *Chlamydiaceae* and indication of typical hosts. The new taxonomy of the family *Chlamydiaceae* within the order *Chlamydiales*. Typical hosts are indicated at the right side; human pathogenic strains are highlighted. The length of lines does not correspond to actual evolutionary distance. After: Bush and Everett, 2001; Everett, et al., 1999.

reside within amoebae. However, their involvement in human infections has been discussed (Heinz, et al., 2007; Friedman, et al., 2003; Horn, et al., 2000).

1.1.2 Developmental cycle of chlamydiae

The obligate intracellular bacteria of the family *Chlamydiaceae* all reside within a membrane-bound vacuole, termed the chlamydial inclusion, inside the host cell. Chlamydiae possess a unique biphasic life cycle, changing between two morphological distinct stages (Fig. 1.2). These are an infectious but metabolically inactive form (elementary body, EB), which never divides, and a replicating non-infective form (reticulate body, RB). The EBs have condensed DNA and are approximately 0.3 µm in size. EBs are released from infected host cells at the end of the cycle and infect new cells, in which they transform into RBs with a diameter of approximately 1 µm and multiply. Chlamydiae usually infect cells that are not actively phagocytic (Moulder, 1991; Matsumoto, 1973; Hammerschlag, 2002; Abdelrahman and Belland, 2005).

As chlamydiae are obligate intracellular pathogens, entry into mucosal epithelial cells is necessary for survival and replication. Attachment of the infectious EBs to the host cells involves electrostatic binding (Stephens, et al., 2001; Su, et al., 1996). Chlamydiae use multiple possible routes of entry. Reports include phagocytosis, clathrin- and lipid microdomain-dependent entry (Hybiske and Stephens, 2007; Dautry-Varsat, et al., 2005; Abdelrahman and Belland, 2005). EBs are equipped with a functional type III secretion system delivering effectors into the host cell, which facilitate bacterial entry (Abdelrahman and Belland, 2005; Clifton, et al., 2004).

The bacteria stay within the membrane-bound phagosome, which is termed the chlamydial inclusion. EB differentiation into the growing form (RB) is accompanied by enlargement and loss of infectivity. Chromosome decondensation and transcription take place. Early genes include genes involved in inclusion modification and nutrient acquisition, e.g. lipids from the host's Golgi vesicles. Within two hours after entry, the inclusion locates to the perinuclear region and remains close to the Golgi apparatus. Chlamydiae actively modify the inclusion membrane to protect their replicative niche and evade the host endocytic pathway, which would lead to fusion with lysosomes (Abdelrahman and Belland, 2005; Hammerschlag, 2002; Hackstadt, et al., 1996).

RBs grow rapidly and replicate through binary fission during the middle of the replication cycle. 24 – 48h post infection RBs differentiate asynchronously back into EBs. 48 – 72h post infection bacteria leave the host cell via cell lysis or exocytosis of the whole inclusion (Abdelrahman and Belland, 2005; Hammerschlag, 2002).

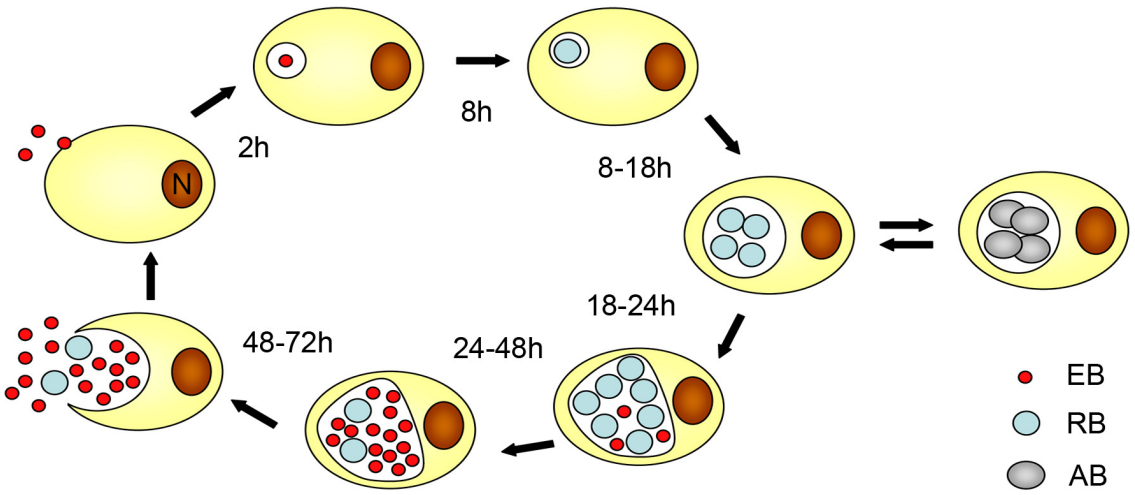


Fig. 1.2: Developmental cycle of chlamydiae. Schematic representation of the chlamydial developmental cycle within host cells (yellow). For description see text. EB: elementary body (red); RB: reticulate body (blue); AB: aberrant body (grey); N: nucleus (brown). After: Hammerschlag, 2002.

All chlamydial species are able to cause persistent infections, which are prolonged and in which the pathogen is not eliminated. Persistent infection can be triggered by antibiotics, cytokines, or nutrient limitation (Harper, et al., 2000; Beatty, et al., 1994; Abdelrahman and Belland, 2005). Under these conditions, the developmental cycle is interrupted and RBs transform into an enlarged aberrant form (aberrant body, AB) with reduced metabolic activity. Persistent ABs often are unsusceptible to antibiotics. Chlamydiae can persist in the host for months to years. This results in continued damage to the host. Persistence can redevelop into open disease, when the limiting conditions change (Abdelrahman and Belland, 2005; Hammerschlag, 2002).

1.2 Chlamydial diseases

Chlamydiae can infect mucosal tissues of humans, mammals and birds causing infections of the eyes, the respiratory and urogenital tract. In addition, chlamydiae have been associated with chronic diseases such as arthritis and atherosclerosis (Gérard, et al., 2010; Vainas, et al., 2009).

Importantly, chlamydiae are the leading cause of infectious blindness worldwide and of the most common bacterial sexually transmitted diseases (STD). In Germany, in average 6 % of patients were infected with chlamydiae as observed in a six year surveillance study (Robert Koch Institute, 2010; http://www.rki.de/cln_160/nn_1759378/DE/Content/Infekt/EpidBull/Archiv/2010/03/Tabelle.html?__nnn=true). Therefore, analysis of chlamydial pathogenesis is of great medical importance.

1.2.1 Medical importance of *Chlamydia trachomatis*

Chlamydia trachomatis is classified into three groups of serovars (A-C, D-K and L1-L3) according to the different diseases they cause. The serovars A – C are the agents of the ocular disease trachoma, an infection of the conjunctiva and inner side of eyelids, eventually causing the eyelids to turn inwards. The eyelashes scratch the cornea, which leads to visual impairment and results in permanent blindness. Trachoma is the most frequent infectious cause of blindness worldwide and is endemic in over 50 countries, predominantly Africa, Middle East and Asia. Approximately 1.3 million people are blinded by trachoma, which makes 3.6 % of total blindness worldwide. In some parts of developing countries, over 90 % of the population is infected (Burton and Mabey, 2009; Resnikoff, et al., 2004; WHO, http://www.who.int/vaccine_research/diseases/soa_std/en/index1.html). *Chlamydia trachomatis* ocular serovars have additionally been associated with the chronic disease arthritis (Gérard, et al., 2010).

C. trachomatis of the second group, serovars D – K, causes urogenital tract infections in woman and men and is associated with the most common STD. There are around 90 million new cases of infections per year worldwide with 4-5 million new cases alone in the USA (WHO, http://www.who.int/vaccine_research/diseases/soa_std/en/index1.html). A high portion of infections stays asymptomatic and thus often untreated. Symptomatic patients present with vaginal discharge or pelvic pain. Infections in woman include cervicitis, salpingitis (inflammation of fallopian tube), and pelvic inflammatory disease (PID) (Ridgway, 1986; Hare, 1983). Tissue damage and scarring occur, whose causing mechanisms are not fully understood, and sequelae include tubal occlusion, ectopic pregnancy and infertility (Darville and Hiltke, 2010; Hare, 1983). Infections in men can result in urethritis and prostatitis (Ridgway, 1986).

The systemic disease lymphogranuloma venereum (LGV) is caused by *C. trachomatis* serovars L1 – L3 and is more common in tropical countries. Unlike other forms of chlamydial infection, in LGV lymph tissue is invaded rather than only epithelial cells. Bacteria enter through primary lesions, e.g. in the vagina or penis, and cause swelling of lymph nodes. Infections can lead to necrotic lesions and scarring, rectal stricture and elephantiasis (thickening of legs or genitals) (Ridgway, 1986; Schachter and Osoba, 1983).

1.2.2 Infections by other chlamydial strains

Chlamydophila pneumoniae, formerly known as *Chlamydia pneumoniae*, is a human pathogen and primarily infects the mucosal epithelium of the respiratory tract causing pneumonia. Symptoms range from mild, self-limiting disease to severe forms. *C.*

pneumoniae is also associated with the chronic diseases arthritis and atherosclerosis (Burillo and Bouza, 2010; Gérard, et al., 2010; Blasi, et al., 2009).

The other species of the *Chlamydiaceae* infect animals (compare Fig. 1.1) but have the potential of zoonotic infections. Especially *Chlamydophila psittaci*, which naturally resides in birds, can cause pneumonia in humans. The disease in humans is called psittacosis or ornithosis (Gregory and Schaffner, 1997). Other bacteria of this family are agents of infertility and abortion, which in sheep, goats, cattle and swine are caused by *Chlamydophila abortus*, *Chlamydophila pecorum* and *Chlamydia suis* (Mohamad and Rodolakis, 2010; Nietfeld, 2001; Hoelzle, et al., 2000). *Chlamydia muridarum* is an agent of pneumonia and infertility in mice (Swenson and Schachter, 1984; Woodland, et al., 1983), while *Chlamydophila felis* and *Chlamydophila caviae* are associated with conjunctivitis in cats and guinea pigs, respectively (Hartmann, et al., 2010; Helps, et al., 2005; Lutz-Wohlgroth, et al., 2006).

1.2.3 Effects of chlamydiae on fallopian tubes

Infection with chlamydiae is closely linked to the pathology of fallopian tubes. The highest risk for tubal pathology was observed in women with antibodies against *C. trachomatis* in combination with increased levels of a marker for inflammation (C-reactive protein), thus being a marker for persistent infection (Land and den Hartog, 2006). Antibody levels against *Chlamydia* are quantitatively related to the severity of fallopian tube damage in infertile women (El Hakim, et al., 2010).

Chlamydia contributes to tubal pathology in several aspects. Chlamydial infections exert a cytotoxic effect on fallopian tube epithelial cells and cause tissue destruction, including loss of microvilli, rapture of cells and cell junctions (Cooper, et al., 1990; Hvid, et al., 2007). The immune response following the infection is believed to lead to tissue scarring.

Pelvic inflammatory disease (PID) is an inflammation of the genital tract ranging from the cervix to the fallopian tubes and ovaries. PID is caused by infection, mostly a sexually transmitted infection, with bacteria such as *Chlamydia trachomatis* or *Neisseria gonorrhoeae* (Soper, 2010). Therefore, repeated chlamydial infections are a risk factor for PID. Importantly, PID is linked to inflammation of fallopian tubes, which can result in scarring and thus chronic pelvic pain, infertility and ectopic pregnancy (Soper, 2010). Over 98% of all ectopic pregnancies, i.e. pregnancies outside of the uterus, are located in the fallopian tubes. Previous PID and damage of the fallopian tubes (e.g. by infection with *Chlamydia trachomatis*) belong to the main risk factors of ectopic pregnancy and tubal factor infertility (Shaw, et al., 2010; Shao, et al., 2010).

Persistent infections by *C. trachomatis* are prevalent in the fallopian tube, despite antibiotic treatment. In addition to PID, subclinical salpingitis is very common. Today,

women frequently delay the wish to get pregnant in the Western world, which often prolongs the time until fertility problems are detected (Mårdh, 2004).

The transport of the human egg and embryo through fallopian tubes to the uterus involves ciliary beating, muscle contraction and flow of tubal secretions. Ciliary activity seems to be central for the transport (Lyons, et al., 2006). Occlusion of the fallopian tubes is thought to eventually inhibit egg transport. Alterations of cilia activity or muscle contraction in the fallopian tube are thought to contribute to ectopic pregnancy and infertility by interfering with egg transport as well (Shao, et al., 2010). Inflammation, as it follows chlamydial infection, is associated with deciliation (Lyons, et al., 2006). Additionally, damage of mainly ciliated epithelial cells was reported (Hvid, et al., 2007). Furthermore, muscular activity can be influenced by chlamydiae, too, as infection with *Chlamydia muridarum* disrupted spontaneous contractions of fallopian tubes in a mouse model (Dixon, et al., 2009).

In addition, the infection with *C. trachomatis* was recently reported to increase expression of a cell surface receptor (prokineticin receptor 2) in human fallopian tubes that is increased in fallopian tubes from women with ectopic pregnancy. Thereby, the infection could generate a microenvironment susceptible for ectopic implantation (Shaw, et al., 2011).

Many ovarian and peritoneal carcinomas originate in the fallopian tubes (Seidman, et al., 2010). Chronic inflammation was shown to be associated with carcinogenesis in some tissues and a source for chronic inflammation of the fallopian tubes is infection with *Chlamydia trachomatis*. The probability of having ovarian cancer was reported to be increased in women with high antibody levels against chlamydial elementary bodies (Ness, et al., 2003). Based on the study by Ness, et al., Carvalho and Carvalho (2008) hypothesized that fallopian tube infection with *C. trachomatis* and associated chronic inflammation may be involved in fallopian tube carcinogenesis that is thought to contribute to some ovarian cancers.

1.3 Chlamydiae and apoptosis

One of the hallmarks of chlamydial infection observed in cell culture is inhibition of apoptosis, presumably to assure intracellular replication. Therefore, chlamydial strains were compared in this study regarding their influence on apoptosis.

Apoptosis is a form of programmed cell death. The controlled deletion of cells is an important mechanism of cell turnover in tissue homeostasis and during embryonic development. Apoptosis is characterized by distinct morphological changes within the cell: condensation of the nucleus, cell shrinkage, nuclear fragmentation, and budding of apoptotic vesicles from the cell surface. These apoptotic bodies contain all nuclear and

cytoplasmic components with the organelles being tightly packed but intact. No cellular components are released into the extracellular environment. Apoptotic bodies are phagocytosed by macrophages, parenchymal cells or epithelial cells, and degraded (Kerr, et al., 1972; Monks, et al., 2005).

1.3.1 Apoptotic pathways

Different pathways of apoptosis induction are known. Mainly the intrinsic pathway via mitochondria and the extrinsic pathway triggered by extracellular signals via a cell surface receptor are involved. In addition, apoptosis can be initiated by cytotoxic T-cells. Various effector proteins, activators and inhibitors of apoptosis are known. The balance between pro- and anti-apoptotic proteins within the cell can determine the cell's fate (Sharma and Rudel, 2009).

The key effector proteins of apoptosis are cysteine-dependent aspartate-specific proteases (caspases). These proteases are produced as inactive zymogens and activated by autocatalysis or cleavage by other caspases. In response to pro-apoptotic signals the caspase cascade is activated, including the key effector caspase-3, and irreversibly leads to cell death. Cell disassembly by caspases is achieved through inactivation of inhibitors of apoptosis (e.g. Bcl-2 proteins), cleavage of cell structures (e.g. the nuclear lamina or cytoskeleton components such as cytokeratin-18) and deregulation of protein activity by cleavage (Schutte, et al., 2004; Thornberry and Lazebnik, 1998; Cohen, 1997).

Another cellular component involved in the apoptotic pathways is the mitochondria. Permeabilization of the mitochondrial membrane is a pro-apoptotic step and releases cytochrome c for the downstream activation of the caspase cascade. The pro-apoptotic BH3-only proteins lead to this permeabilization (Sharma and Rudel, 2009; Adams and Corey, 1998).

As caspase activation irreversibly leads to cell death, it must be strictly regulated. The inhibitor of apoptosis proteins (IAPs) are able to directly bind and inhibit some of the caspases (Huang, et al., 2001; Deveraux, et al., 1997). Proteins of the Bcl-2 family promote cell survival by interacting with and inhibiting proteins required for permeabilization of the mitochondrial membranes (Adams and Corey, 1998).

1.3.2 Influence of chlamydiae on apoptosis

Apoptosis plays a role in protection against pathogens. It eliminates the infected cell and as all intracellular content of the dying cell is present in the apoptotic bodies, microbial antigens are transported to uninfected antigen-presenting cells. Obligate intracellular pathogens, such as *Chlamydia* and *Rickettsia*, therefore need to block apoptosis in order

to replicate inside the host cell and to circumvent immune recognition (Böhme and Rudel, 2009; Sharma and Rudel, 2009).

Chlamydiae are widely described to inhibit apoptosis within their host cells (Böhme, et al., 2010; Häcker, et al., 2006; Rajalingam, et al., 2001). Apoptosis resistance has been reported for all major chlamydial species, including *Chlamydophila pneumoniae* and *Chlamydia trachomatis* (Sharma and Rudel, 2009). An initial study showed that only infected cells were protected (Rajalingam, et al., 2001).

Chlamydiae were shown to block the mitochondrial permeabilization and cytochrom c release by degrading the pro-apoptotic BH3-only proteins. This in turn inhibits the activation of the downstream caspases, including caspase-3 (Pirbhai, et al., 2006; Ying, et al., 2005; Fischer, et al., 2004; Fan, et al., 1998). Additionally, anti-apoptotic proteins of the Bcl-2 family (e.g. Mcl-1) are upregulated (Hess, et al., 2001). Chlamydiae also interfere with the apoptosis pathways downstream of mitochondria. Some inhibitor of apoptosis proteins (IAPs) are upregulated in cells infected with chlamydiae, other IAPs are stabilized, leading to inhibition of caspase-3 (Rajalingam, et al., 2006; Hess, et al., 2001).

In case of *Chlamydia trachomatis*, studies were mainly performed with the serovar L2, which is a widely used laboratory strain, or in some studies with ocular serovars (Böhme, et al., 2010; Rajalingam, et al., 2006; Ying, et al., 2005; Fischer, et al., 2004; Fan, et al., 1998). Apoptosis resistance of cells infected with Clamydiae is thought to play a role in establishing long-term interactions and persistence, which is associated with chronic infections (Byrne and Ojcius, 2004).

Chlamydiae were described to act pro-apoptotically in the late stages of the infection cycle prior to escape from the host cells. In addition, some reports describe cell death induced by chlamydiae, which was, however, later characterized to be different from apoptosis (Sharma and Rudel, 2009; Byrne and Ojcius, 2004).

1.4 Primary cells and tissue

Chlamydiae infect the urogenital tract and can cause infertility in woman (see chapter 1.2). Various model systems exist to study these infections. However, they can not completely simulate the complex situation of the primary tissue within organs and might lack important factors present *in vivo*. As primary cells and tissue of the human fallopian tube (FT) are targets of chlamydial infection, primary FT cells and FT *ex vivo* tissue culture were used in this study.

1.4.1 Anatomy of the female genital tract

The female reproductive tract contains pairs of ovary and fallopian tube (also named oviduct or tuba uterina). The ovary containing the egg cells is in close proximity to the fallopian tube, which connects to the uterus and performs the task of transporting the egg into it (Fig. 1.3).

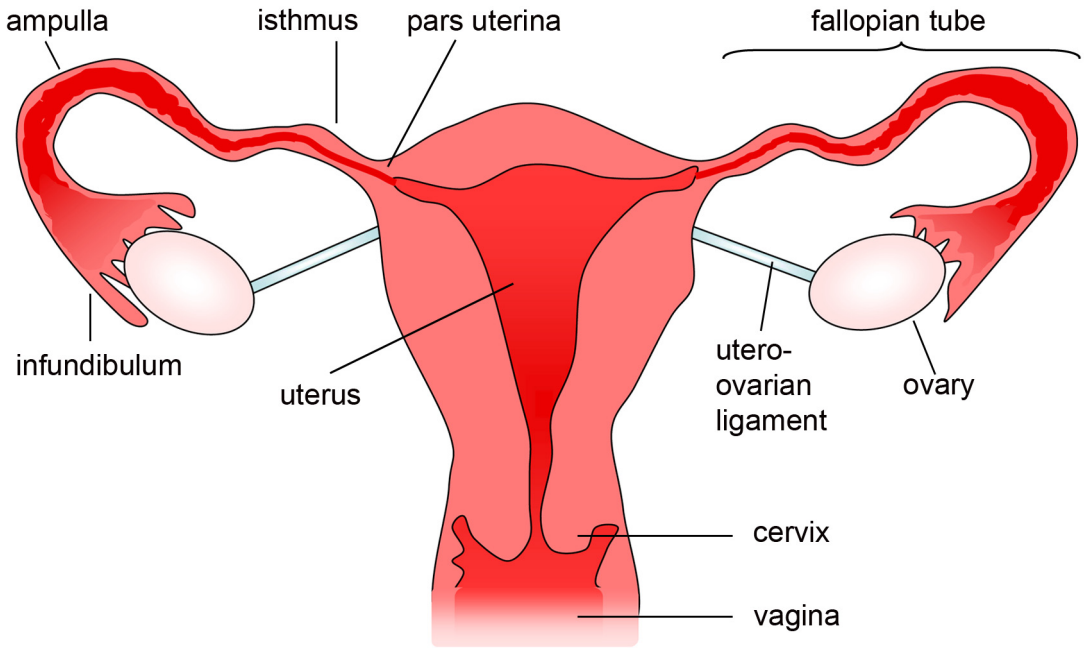


Fig. 1.3: Anatomy of the human female genital tract. The fallopian tube connects the ovary with the uterus. The ovary is held in place by the utero-ovarian ligament. The cervix protrudes into the vagina. The fallopian tube consists of four segments, which differ in width and extent of mucosal folds: the infundibulum, the ampulla, the isthmus and the pars uterina. For further description see text.

The fallopian tube comprises distinct regions varying in their structure. The infundibulum is a cone-like structure with fimbriae and an opening towards the ovary. It absorbs the egg. The ampulla is the longest part of the tube and the one with the largest lumen. The mucous inner layer, the mucosa, forms multiple folds in the ampullary region (Fig. 1.4). The following part, the isthmus, is located close to the uterus and has a narrowed lumen (Fig. 1.5). The mucosal folds are reduced, and muscle layers are thicker than in the previous parts. The opening into the uterus lumen runs inside the uterus wall and is named pars uterina (or pars uterina tubae uterinae). The endometrium is the mucosa (see below) of the uterus. The neck of the uterus, the cervix, protrudes into the vagina (Nasu and Narahara, 2010; Saksouk and Johnson, 2004; Bucher and Wartenberg, 1997).

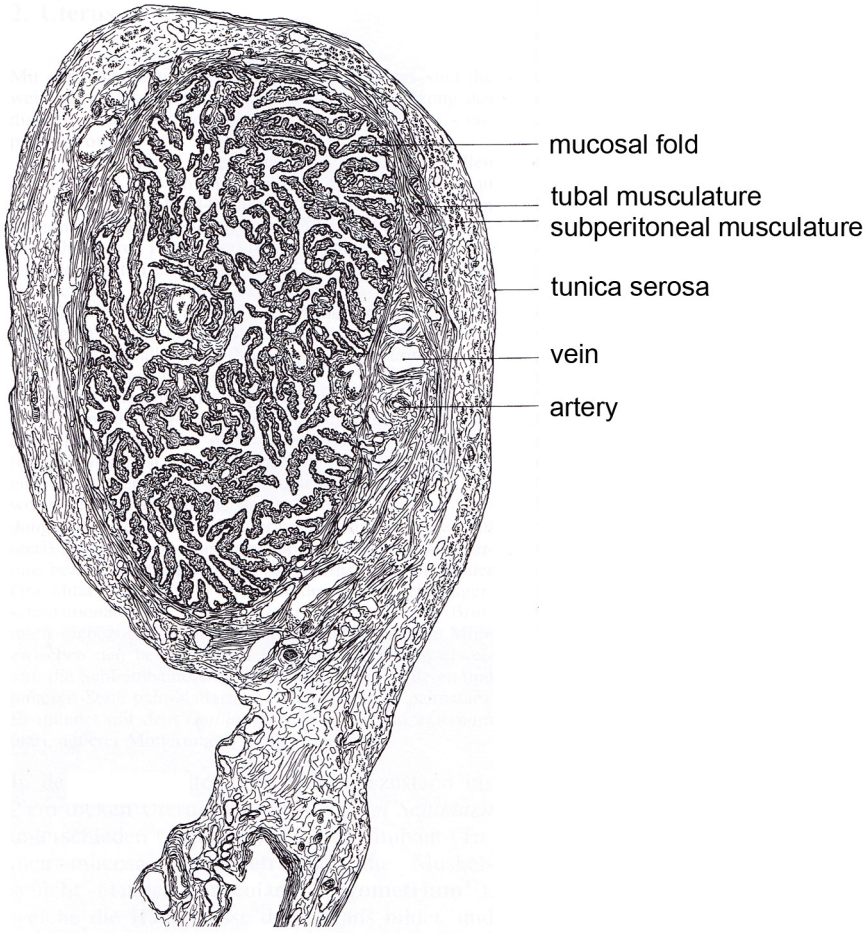


Fig. 1.4: Transversal section through the ampullary region of a fallopian tube. Ampullary region of a 20-year-old woman. Extensive mucosal folds protrude into the lumen of the tube; the tubal musculature contains blood vessels; subperitoneal musculature surrounds the tube and is encircled by the tunica serosa (lining of the abdominal cavity). Drawing of hematoxillin-eosin staining. From Bucher and Wartenberg, 1997.

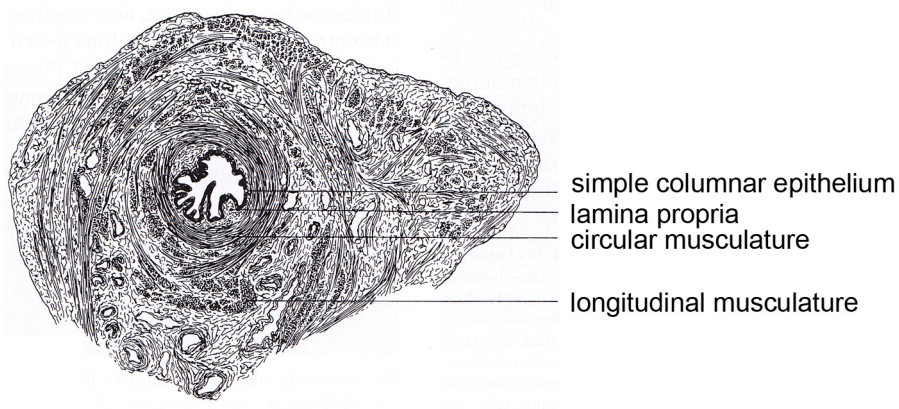


Fig. 1.5: Transversal section through the isthmus of a fallopian tube. Isthmic region near the uterus of a 44-year-old woman. Simple columnar epithelium lines the lumen of the tube; the extent of mucosal folds is reduced; circular and longitudinal musculature, the two layers of tubal musculature, form thick layers. Drawing of hematoxillin-eosin staining. From Bucher and Wartenberg, 1997.

1.4.2 Epithelia and endothelia

Epithelia are the cell layers lining all surfaces of the body. This includes the inner surfaces of organs, the internal body cavities and the mammalian skin. Epithelial cells are in close proximity to each other and form a closed cell layer. The basement membrane, a thin fibrous layer beneath the epithelial cells, anchors the basal side of the epithelium to the underlying connective tissue. The apical side of the cells faces the lumen of an organ, a body cavity or the environment (in case of skin). In addition, a specialised type of epithelium exits, named endothelium, which lines the inner surfaces of blood and lymph vessels (Bucher and Wartenberg, 1997).

Classification of epithelia is based on cell shape and number of layers. Single-layer epithelium is termed simple, whereas epithelium consisting of two or more cell layers is named stratified. According to the height of cells in the top layer, epithelia are classified as columnar (with cells taller than wide), cuboidal (with cube-like shaped cells) or squamous (with flattened cells) (Bucher and Wartenberg, 1997).

The mucosa of the human fallopian tube consists of a single-layer columnar epithelium and the connective tissue underneath (lamina propria; also named lamina propria mucosae) (Fig. 1.6). Ciliated as well as non-ciliated cells are present in the epithelial layer (Bucher and Wartenberg, 1997). The cytoskeletal intermediate filaments cytokeratin-8 and cytokeratin-18 are typically found in simple epithelium (Moll, 1993).

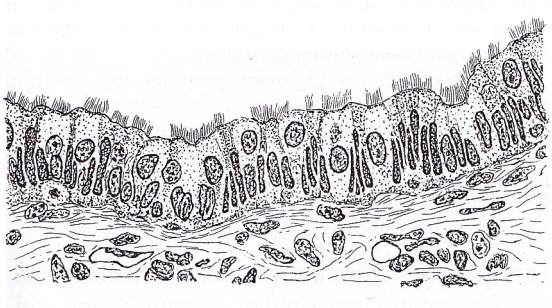


Fig. 1.6: Mucosa of the human fallopian tube. The mucosa of the human fallopian tube consists of a single-layer columnar epithelium (with ciliated and non-ciliated cells) and the lamina propria underneath. Drawing of hematoxillin-eosin staining. From Bucher and Wartenberg, 1997.

Polarized epithelial cells

In the body, epithelial layers form a tight barrier. The cells are polarized possessing distinct membrane compartments with differential composition: the apical membrane (AM) and the baso-lateral membrane (BLM) (Fig. 1.7). Tight junctions (TJ) are the protein complexes responsible for barrier function. They consist among others of proteins of the claudin-family as well as occludin and ZO-proteins (Furuse, 2010; Marshall, et al., 2009; Radisky, 2005).

Adherens junctions mediate cell-cell-adhesion via homophilic interactions of cadherins (Fig. 1.7). These possess a transmembrane and a surface part. Cadherin-mediated contacts depend on Ca^{2+} interacting with the extracellular domains. E-cadherin is the form predominantly found in epithelial cells and VE-cadherin was described as vascular endothelial cadherin. The intracellular catenins connect the junctions to the actin cytoskeleton (Meng and Takeichi, 2009; Radisky, 2005).

Additional proteins mediating adherence between cells are the homophilic transmembrane proteins EpCAM (epithelial cell adhesion molecule, CD326), which is specifically expressed in epithelial cells, and PECAM-1 (platelet/endothelial cell adhesion molecule 1, CD31) in endothelial cells (Baeuerle and Gires, 2007; Woodfin, et al., 2007). EpCAM was also described to interact directly with the tight junction protein claudin-7 as well as to antagonize cell contacts established by cadherins (Ladwein, et al., 2005; Litvinov, et al., 1997). The contact between the basal membrane of the epithelial cells and the basement membrane is mediated by a group of transmembrane proteins, the integrins (Radisky, 2005).

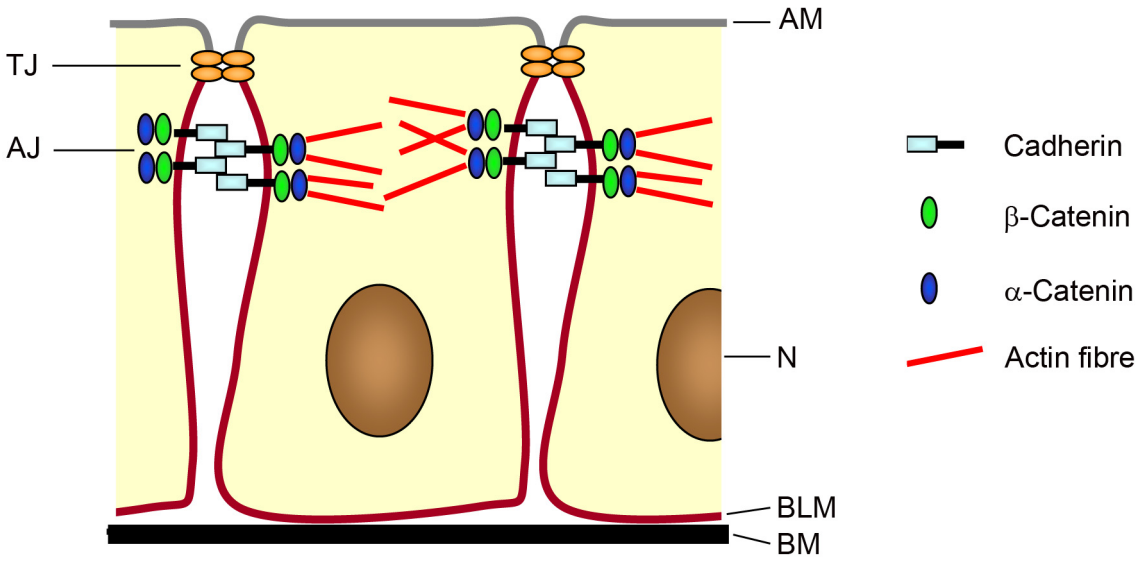


Fig. 1.7: Tight and adherens junctions in polarized epithelial cells. For description see text. AJ: adherens junction; AM: apical membrane (grey); BLM: baso-lateral membrane (dark red); BM: basement membrane (black); N: nucleus (brown); TJ: tight junction.

1.5 Immortalization of primary cells

Isolated primary cells have a confined life span and depending on the cell type do not grow in cell culture in many cases. In order to generate a stock of cells growing continuously, cellular characteristics have to be changed. However, the least aggressive

approach should be used to acquire least changes in the cells. Immortalization of primary cells has been developed as widely-used tool.

1.5.1 Immortalization and transformation

Targeted immortalization of cells is assumed to lead to more reliable results than using transformed cells, as immortalization is intended to retain properties of primary cells. Immortalized cells possess infinite life span. Unlike transformed cells, however, other features of primary cells can be kept. This includes anchorage-dependent non-invasive growth and contact inhibition of growth. In addition, targeted immortalization aims to avoid tumorigenicity of cells.

In contrast, transformation is a process, by which cells acquire infinite life span and anchorage-independent and invasive growth. Contacts change between cells and adhesion to substrate or basement membrane is disrupted. The transformed cells loose contact inhibition of growth and tumor formation can occur. Carcinogenic transformation leads to the unlimited, often invasive growth of cancer cells as well as tumor formation, tissue invasion and metastases. In addition, cancer cells evade apoptosis, are self-sufficient in growth signals and insensitive to anti-growth signals. Cancer tissue sustains angiogenesis (Lin, et al., 2011; Lapierre, et al., 2011; Hanahan and Weinberg, 2000).

Transformation is a multistep process that is believed to require alterations in at least three cellular pathways. Key tumor suppressors (such as p53 and Rb) need to be inactivated, cell proliferation has to be activated and cell aging connected to chromosome replication has to be prevented (Sun, et al., 2005; Hahn, et al., 1999).

The tumor suppressor p53 is a transcription factor acting as master regulator, as it influences the expression of an array of genes. Some of these genes are involved in DNA damage repair pathways and induction of cell cycle arrest. p53 is activated by cellular stress (Menendez, et al., 2010). Other well known tumor suppressors are the retinoblastoma proteins (Rb), which inhibit DNA replication in resting cells. These proteins bind transcription factors that are needed for the activation of the DNA replication (Levine, 2009).

Ras proteins are involved in the regulation and activation of cell proliferation. These small GTPases are part of the signal transduction from cell surface receptors to several pathways within the cells, including the mitogen activated protein kinase (MAPK) pathway. Mutations can lead to permanently activated Ras, resulting in activation of cell proliferation and survival (Frémin and Meloche, 2010). Furthermore, Mizumoto, et al. (2006) demonstrated that immortalized non-tumorigenic cells with activated telomerase and inactivated Rb were rendered invasive and thus tumorigenic upon introduction of an active Ras mutant.

Activation of the human telomerase, which is not active in most cells of the body, can prevent cell aging. In absence of the telomerase activity, the telomeres at the end of the chromosomes are shortened during DNA replication. This process has been connected to cellular senescence. The activation of telomerase results in the elongation of telomeres and therefore antagonizes cell aging (see chapter 1.5.2).

Oncogenes discovered in cancers can induce cell growth. Therefore, these oncogenes were used for targeted immortalization of cells. In the literature, the simultaneous usage of two oncogenes together is often described.
In order to have more relevant models for the investigation of chamydial infection, cell lines were generated within this study. The oncoproteins used to immortalize cells in this study are hTERT, SV40T and Bmi1. The characteristics of these oncoproteins are summarized in table 1.1 and described in detail below, including their cellular functions, occurrence and usage for immortalization of primary cells (chapters 1.5.2, 1.5.3, and 1.5.4). Lentivirus-mediated gene transfer of oncogenes was used in this study for the targeted immortalization, which is described in detail below (chapter 1.6).

Tab. 1.1: Functions and reported usage of hTERT, SV40T and Bmi1. For references, see text.

Oncoprotein	Function	Immortalized human cells
hTERT (human telomerase reverse transcriptase)	- catalytic subunit of human telomerase - elongation of telomeric repeats at end of chromosomes - prevention of progressive telomere shortening, a factor of senescence	- urothelial cells - meibomian gland epithelial cells - esophageal epithelial cells - liver endothelial cells - muscle satellite cells
SV40T (Simian Virus 40 large tumor antigen)	- inactivation of retinoblastoma proteins, which leads to activation of transcription factors and entry into S phase of cell cycle - inactivation of p53 tumor suppressor - protein necessary for the viral DNA replication and transcription	- renal epithelial cells - mammary epithelial cells - bile duct epithelial cells - endothelial progenitor cells - liver endothelial cells - hepatocytes
Bmi1 (B lymphoma Mo-MLV insertion region 1)	- transcriptional repressor - repression of the ink4a-locus with the tumor suppressor genes p16 and p19Arf (human homolog: p14Arf) - a polycomb ring finger protein - required for self-renewal of adult stem cells	- bronchial epithelial cells - nasopharyngeal epithelial cells - mammary gland epithelial cells - mammary epithelial cells - olfactory ensheathing glia - muscle satellite cells - hepatocytes

1.5.2 hTERT

The human telomerase reverse transcriptase (hTERT) is the catalytic subunit of the telomerase. This enzyme elongates the telomeres at the end of the chromosomes through addition of a repeating pattern of six nucleotides (TTAGGG) (Morin, 1989).

Telomerase is a ribonucleoprotein complex consiting of RNA and protein components, which are both required for activity. The telomerase has reverse transcriptase activity and uses the RNA as template. This enzyme is able to elongate the single stranded DNA of the telomeres which end with a 3' DNA overhang (Greider and Blackburn, 1987; Shippen-Lentz and Blackburn, 1990; Blackburn, et al., 1989).

Generally, telomerase is repressed in somatic cells. This leads to telomere shortening during aging of cells (Harley, et al., 1990). Thus, telomere expression is a factor of cellular senescence. Deregulation of telomerase expression may play a role in oncogenesis as telomerase is reactivated in most advanced cancers (Artandi and DePinho, 2010). Telomerase activity was detected in approx. 87 % of ovarian epithelial cancers and hTERT was significantly stronger expressed in renal cell carcinoma compared to the adjacent tissues (Zhang F, et al., 2008; Wu, et al., 2008). hTERT expression is also upregulated in cervical and gastric cancer (Xi, et al., 2005; Jong, et al., 1999; Gigek at al., 2009).

As hTERT expression is rate-limiting for telomerase activity, inserting the hTERT oncogene into primary cells has been widely used for cellular immortalization. Successful immortalization was reported e.g. for human urothelial cells, human meibomian gland epithelial cells, a human esophageal epithelial cell line, human liver endothelial cells and human muscle satellite cells (Chapman, et al., 2008; Liu, et al., 2010; Cheung, et al., 2010; Matsumura, et al., 2004; Cudré-Mauroux, et al., 2003). Usage of hTERT led to cell lines with long-term genotypic and phenotypic stability, nearly-diploid and non-tumorigenic cells, with only sometimes genetic and genomic alterations found (Cudré-Mauroux, et al., 2003; Cheung, et al., 2010).

1.5.3 SV40T

The Simian Virus 40 large tumor antigen (SV40T) is a viral protein from Simian Virus 40, a non-enveloped dsDNA virus of the polyomavirus group. SV40T is expressed early in the viral lifecycle. It binds to the viral origin of replication and is required for the initiation of replication and for transcription of the viral DNA (Fanning and Zhao, 2009).

In the host cells, SV40T additionally binds to and inactivates the retinoblastoma (Rb) proteins. This interaction eliminates Rb-E2F repressive complexes and activates transcription factors including E2F and thereby finally leads to entry of the cells into the cell cycle phase of DNA synthesis (S phase). This is necessary for the virus to replicate

its own DNA. SV40T also binds to and inactivates the p53 tumor suppressor (Levine 2009; Cantalupo, et al., 2009; Sáenz-Robles, et al., 2007). In addition, SV40T can inhibit apoptosis (Yu and Alwine, 2002).

The SV40T mediated gene regulation was shown to be primarily exerted through and dependent on the binding to Rb proteins (Rathi, et al., 2009). SV40T induces tumors in animals and can transform many types of cells in culture (Butel and Lednicky, 1999). Among these are human renal epithelial cells, human mammary epithelial cells, human cholangiocytes, which are epithelial cells of the bile duct, as well as human endothelial progenitor cells, human liver endothelial cells and human hepatocytes (Kowolik et al, 2004; Garbe, et al., 1999; Maruyama, et al., 2004; Qiu, et al., 2006; Matsumura, et al., 2004; Nguyen, et al., 2005).

SV40T was reported to be very efficient for growth induction of cells, but led to chromosomal aberrations and strong phenotypic changes (Cudré-Mauroux, et al., 2003). SV40T induced chromosomal instability was reported to induce a variety of chromosomal aberrations, the most frequently observed being dicentric chromosomes (chromosomes with two centromeres) and also tetraploidy (Hein, et al., 2009; Ray, et al., 1992).

1.5.4 Bmi1

The B lymphoma Moloney Murine Leukemia Virus insertion region 1 (Bmi1) was first identified as an oncogene that is involved in the formation of mouse lymphomas (van Lohuizen, et al., 1991). A homologous gene exists in humans. Bmi1 has a regulatory role in cell cycle and senescence and is a transcriptional repressor. It targets the ink4a-locus, which encodes the tumor suppressors p16 and p19Arf (murine protein; p14Arf is the human homolog). Therefore, Bmi1 expression leads to the downregulation of the ink4a-locus (Jacobs, et al., 1999).

The mechanisms of Bmi1 action involve epigenetic silencing via histone modifications. As a member of the polycomb repressive complex 1 (PRC1) Bmi1 is located at the ink4a-locus together with other polycomb group proteins in young proliferating cells. The locus is silenced through histone H3 methylation. In contrast, in senescent cells a histone demethylase is expressed and recruited. The polycomb proteins dissociate from the locus, which then is transcriptionally active. The repression exerted by Bmi1 requires direct association with the locus (Agherbi, et al., 2009; Bracken, et al., 2007). Additionally, Bmi1 expression was reported to induce hTERT activity in immortalized cell lines (Song, et al., 2006; Dimri, et al., 2002).

Bmi1 deficient mouse embryonic fibroblasts are impaired in the progression into S phase of the cell cycle and prematurely enter senescence (Jacobs, et al., 1999). Bmi1 is required for the self-renewal of adult haematopoietic stem cells and plays an important

role for the regulation of proliferation of stem and progenitor cells (Park, et al., 2003; Lessard and Sauvageau, 2003).

Bmi1 is upregulated in many cancers, e.g. ovarian cancer and gastric cancer and its misregulation has also been implicated in the development of some types of lung cancer (Yang, et al., 2010; Zhang, et al., 2008; Zhang, et al., 2010; Vonlanthen, et al., 2001). Strong Bmi1 expression could be correlated with advanced tumor stages or shortened patient survival in ovarian and gastric cancers (Yang, et al., 2010; Zhang, et al., 2010).

Bmi1 was described for the immortalization of various cells including human bronchial epithelial cell lines, human nasopharyngeal epithelial cells, human mammary gland epithelial cells and human mammary epithelial cells (Fulcher, et al., 2009; Song, et al., 2006; Haga, et al., 2007; Dimri, et al., 2002). In addition, Bmi1 was used for generation of cell lines of human olfactory ensheathing glia, human muscle satellite cells and human hepatocytes. Overexpression of Bmi1 was also reported to immortalize fibroblasts (García-Escudero, et al., 2010; Cudré-Mauroux, et al., 2003; Nguyen, et al., 2005; Jacobs, et al., 1999). Immortalization of human muscle satellite cells with Bmi1 together with hTERT led to a cell line with long-term genotypic and phenotypic stability (Cudré-Mauroux, et al., 2003).

1.6 Lentiviruses

In this study, we wanted to use lentivirus-mediated transfer of oncogenes for the immortalization of primary cells to generate new cell lines. The lentiviral plasmid system used in this work is based on HIV-1. Therefore, structure and replication of the lentivirus HIV-1 are introduced.

Lentiviruses belong to the family of retroviruses, which mainly infect vertebrates and cause various symptoms, including tumors and immunodeficiencies. Retroviruses contain an RNA-genome and were named after their enzyme “reverse transcriptase”, which can transcribe RNA into double-stranded DNA. The human immunodeficiency virus (HIV) is a human pathogenic lentivirus (Modrow, et al., 2010).

1.6.1 Structure of lentiviruses

Lentiviral particles consist of a capsid surrounded by a coating membrane, which is derived from the cytoplasma membrane of the host cell. The structure of HIV-1 is shown as example (Fig. 1.8). The viral glycoproteins (the external glycoprotein gp120 and the transmembrane protein gp41) are associated with the coating membrane. The matrix proteins are associated with the inner side of the coating membrane and form a net-like protein layer. The viral capsid, also termed the viral core, is located inside the viral particle. Its outer layer is formed by the capsid proteins. The capsid contains two identical

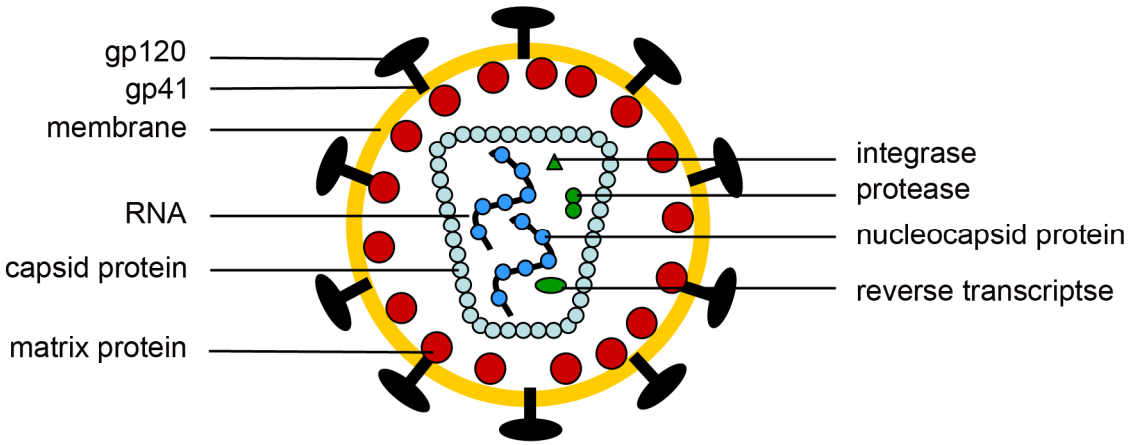


Fig. 1.8: Structure of HIV-1. For description see text. After: Ganser-Pornillos, et al., 2008; Modrow, et al., 2010.

copies of a single-stranded RNA genome. The nucleocapsid proteins form a complex with the RNA genome. In addition, the viral enzymes integrase, reverse transcriptase and protease are located within the capsid (Ganser-Pornillos, et al., 2008; Modrow, et al., 2010).

The genomes of all retroviruses code for three major precursor protein products: Gag (group specific antigens – matrix, capsid, and nucleocapsid), Pol (enzymatic activities) and Env (envelope glycoproteins). The 5'- and 3'-ends of the coding region are flanked by regulatory sequences required for reverse transcription as well as integration into the host's genome (Modrow, et al., 2010).

1.6.2 Replication cycle of lentiviruses

The extracellular glycoprotein mediates adsorption of the viral particle to the host cell. In case of HIV-1, gp120 binds to CD4, which is expressed on the cell surface of immune cells such as T helper cells and macrophages (Modrow, et al., 2010).

The viral and cell membranes fuse. The capsid and its content are the parts of the virus that are released into the cytoplasm of new host cells upon infection. The capsid uncoats to release its content and reverse transcription of the viral RNA into double-stranded DNA takes place (a complex mechanism reviewed in Haseltine, 1991). The proviral DNA is trafficked to the nucleus and integrates into the host cell's genome by action of the viral integrase. The integrated virus is called provirus (Ganser-Pornillos, et al., 2008; Modrow, et al., 2010).

The integrated provirus carries identical sequences at the ends, which are generated during reverse transcription of the viral RNA into double-stranded DNA. These sequences

are termed long terminal repeats (LTR) and flank the viral genes in the same orientation at both sides (Modrow, et al., 2010).

The proviral DNA is transcribed in the host nucleus and translated in the cytoplasm. New copies of the viral RNA genome as well as the viral proteins are generated. The protein and RNA components assemble at the cytoplasma membrane and form immature viral particles budding from the cell (Modrow, et al., 2010). The structural proteins of the HIV-1 virus are derived from the Gag-polyprotein, which is cleaved during virus assembly by the viral protease. The newly cleaved proteins rearrange to form the mature infective virus particle (Ganser-Pornillos, et al., 2008).

1.6.3 Lentiviruses as vectors for genetic transfer

Various viral vector systems are used to transfer genetic material into target cells. Lentiviral vectors, oncoretroviral vectors, adeno-associated virus (AAV) vectors and adenovirus vectors are used for the development of treatment for hereditary disorders. Advantages and disadvantages of the different vector systems for gene transfer are summarized in table 1.2 (O'Connor and Crystal, 2006).

In this study, a lentiviral system was used. Among the advantages of lentivector systems are integration of the transferred genetic material into the chromosome of the target cells, a requirement for long term gene expression, and a relatively large capacity (8-10 kb). In contrast to conventional retroviral vectors (oncoretroviruses), which can only transduce dividing cells, lentiviruses can infect dividing as well as non-dividing cells. Additionally, the genetic information expressing viral structural proteins is not transferred, thus these viral proteins are not made in the host and attack of vector-transduced cells by the immune

Tab. 1.2: Advantages and disadvantages of viral gene transfer systems. For references, see text.

Viral system	Advantages	Disadvantages
Lentiviral vector	- long term expression: gene integration - transduction of non-dividing cells	- risk of insertional mutagenesis - human pathogens
Onco-retroviral vector	- long term expression: gene integration - non-human origin	- only transduction of dividing cells - risk of insertional mutagenesis
AAV vector	- non-pathogenic - transduction of non-dividing cells - no mandatory integration	- limited transgene size
Adenovirus vector	- high cloning capacity	- transient expression

system is unlikely. However, as lentiviral vectors integrate into the target genome, there is a risk of insertional mutagenesis. Lentiviruses are human pathogens, requiring additional safety considerations leading to replication-incompetent viral particles (see below) (O'Connor and Crystal, 2006; Salmon and Trono, 2006; http://tronolab.epfl.ch/).

The lentiviral vector system used in this study is derived from HIV-1 genome and was generated in the laboratory of Didier Trono. For safety reasons the formation of replication-competent viral particles has to be prevented. Therefore, all required sequences are distributed on as many independent units as possible. Unneeded regulatory or coding sequences are deleted from the viral genome. In the system used here the information for the viral enzymes and inner structural proteins, the envelope protein and the genetic information of the target genes, which will be transferred, is separated on three plasmids. Replication-competent viral particles could only occur by multiple recombinations. The system is designed in the way that only the target genes and flanking regulatory sequences enter the infected cells, without any genetic information for the viral enzymes and structural proteins. Thus, the infectious particles can integrate once into the host genome, but not replicate (Salmon and Trono, 2006).

The HIV-1 envelope protein can be exchanged in this system with the analogous protein of another virus. The exchange is called pseudotyping and alters the tropism of the viral particle. Frequently, the envelope glycoprotein G-protein of vesicular stomatitis virus (VSV-G) is used due to its high stability and broad tropism. VSV-G binds to membrane phospholipids ubiquitously expressed in mammalian cells (Salmon and Trono, 2006; Mastromarino, et al., 1987).

Lentivectors are not only used for gene therapy, but also for immortalization of primary cells. They are especially useful, because isolated primary cell populations do not always grow and the lentivectors are able to transduce specific genes into non-dividing as well as dividing cells (Fulcher, et al., 2009; Nguyen, et al., 2005; Cudré-Mauroux, et al., 2003; Salmon, et al., 2000).

The lentiviral plasmid is transcribed and integrated in the same way as described for lentiviruses (see above). The plasmid encoding the target gene to be transferred is transfected into producer cells and transcribed into the genomic viral RNA (Fig. 1.9). Together with the structural and enzymatic components generated from two additionally transfected plasmids this RNA forms the lentivector particles. Once the lentivector particles infected new target cells, reverse transcription takes place inside the target cells and the provirus integrates (Salmon, et al., 2000).

We used a specialized lentiviral system containing the Cre-lox-system. Thereby, this lentiviral system comprises the possibility to excise the integrated target gene from the host chromosomes. For this purpose, a lox-sequence is located on the lentiviral plasmid.

The lox-sequence is a DNA element recognized by the recombinase Cre. As the sequence at one end of the genomic viral RNA gets duplicated during reverse transcription, the two lox-sequences flank the integrated target gene (Fig. 1.9). Cre mediates recombination between these sites leaving a viral LTR sequence in the host genome. The Cre-lox-system was reported to function efficiently (Kowolik, et al., 2004; Salmon, et al., 2000; Westerman and Leboulch, 1996).

During excision of the transferred construct by Cre, the viral thymidine kinase of Herpes Simplex Virus type 1 (HSV-1 TK) is used as suicide gene for negative selection. HSV-1 TK converts a nucleoside analogon (e.g. ganciclovir) to phosphorylated nucleotides. Their incorporation into the DNA blocks replication. Thus, cells still carrying the construct are sensitive to ganciclovir and die (Salmon, et al., 2000; Caruso, 1996).

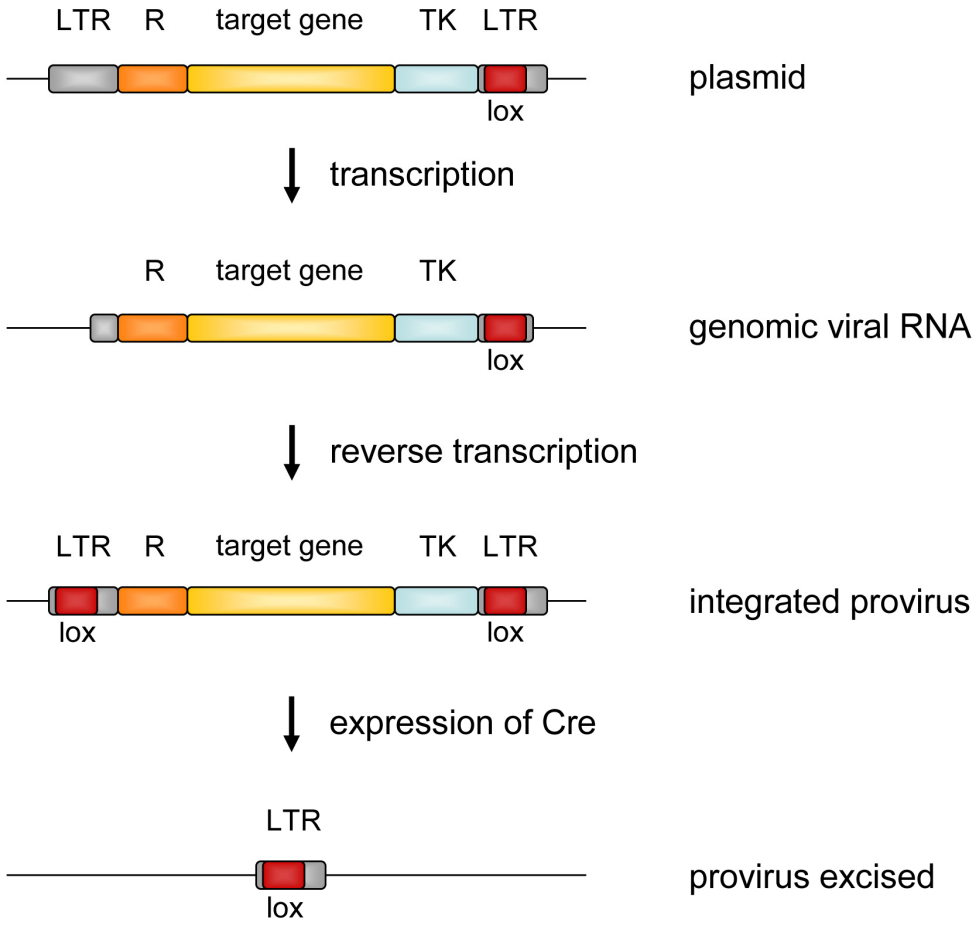


Fig. 1.9: Schematic diagram of the lentivector replication cycle. For description see text. LTR: long terminal repeat; R: regulatory elements; TK: HSV-1 thymidine kinase; lox: lox-sequence. After: Salmon, et al., 2000.

1.7 Objective

Infections caused by chlamydiae can induce several severe diseases in the human population, including blindness, infertility and pneumonia. So far, the widely used infection models comprise cancer-derived cell lines in culture. These simplified models are useful for analysis of single factors in host-pathogen-interaction, but neither represent the primary state of a cell nor the complex infection situation *in vivo* involving multiple cell types.

The aim of this work was to establish new model systems based on human primary cells and tissue culture to investigate chlamydial infections in a context approximating the *in vivo* situation. Primary human cells should be subjected to reversible immortalization by lentivirus-mediated gene transfer in order to generate stable cell stocks. At the same time, reversion of the immortalizing factor would allow to work in cells similar to the primary cells. Epithelial cells from the fallopian tube and endothelial cells from the umbilical vein can serve as model systems for chlamydiae. As neither a reversibly immortalized fallopian tube cell line nor commercially available primary fallopian tube cells existed, establishing of cell isolation and culture should be part of this work prior to immortalization of the primary cells.

As no cell culture model system simulates the complex structure of primary tissues, a second model should be established by directly using *ex vivo* fallopian tube tissue culture. The tissue culture should be implemented for analysis of the course of infection with *Chlamydia trachomatis* including changes occurring within the infected epithelium. In addition, *Chlamydia trachomatis* serovars causing diverse diseases should be analyzed for differences in their effects on the host cells.

2 Materials and methods

2.1 Materials

2.1.1 Primary tissue

Primary healthy human fallopian tube (FT) tissue was obtained from the Clinics for Obstetrics and Gynecology, Campus Virchow-Klinikum and Campus Mitte, at Charité University Hospital, Berlin, Germany, kindly provided by Dr. Christina Fotopoulou and Dr. Kati Hasenbein, with given consent of patients. Material transfer and experimental usage were approved by the ethics commission of the Charité University Hospital (application no. EA1/002/07).

Human umbilical cords were obtained shortly after childbirth from Clinics for Obstetrics and Gynecology, Campus Virchow-Klinikum and Campus Charité Mitte, at Charité University Hospital, Berlin, Germany, kindly provided by Dr. Silke Michaelis (presently Campus Benjamin Franklin) and Dr. Gabriele Gossing after given consent of patients.

2.1.2 Cell lines and primary cells

Tab. 2.1: Description of cell lines and primary cells.

Cells	Source and media
End1 cells	End1/E6E7, human epithelial endocervical cells, originated from primary cells via HPV16 E6E7 immortalization (ATCC, no. CRL-2615), cultivated in DMEM medium
FT cells	human fallopian tube cells, primary and immortalized cells (this study), cultivated in MEM-Earl`s medium
Hela229 cells	human epithelial cells, originated from cervix carcinoma (ATCC, no. CCL-2.1), cultivated in RPMI-1640 medium
Hela05 cells	human epithelial cells, originated from cervix carcinoma (DSMZ, no. ACC57; obtained from ATCC, no. CCL-2), cultivated in RPMI-1640 medium
HUVEC cells	human umbilical vein endothelial cells, primary and immortalized cells (this study), cultivated in ECGM medium
T84 cells	human colon epithelial cells, derived from lung metastasis of colon cancer (ATCC, no. CCL-248), cultivated in DMEM/Ham's F12 medium
TMNK-1 cells	human liver endothelial cell line, immortalized via hTERT and SV40T (Matsumura, et al., 2004), cultivated in DMEM medium, kindly provided by Dr. Naoya Kobayashi (Okayama University, Japan)
293T cells	human embryonic kidney cells (HEK 293T, ATCC, no. CRL-11268), cultivated in DMEM II medium

2.1.3 Bacteria

Chlamydia trachomatis Lymphogranuloma venereum (LGV) serovar L2 (ATCC, VR-902B)

Chlamydia trachomatis serovar D (ATCC, VR-885)

2.1.4 Cell culture media, supplements and buffers

Tab. 2.2: Basal media.

Medium	Description
RPMI-1640	with HEPES (Gibco-Invitrogen, no. 52400-041), supplemented with 2 mM L-glutamine
DMEM	Dulbecco`s minimal essential medium (Gibco-Invitrogen, no. 10938-025), supplemented with 2 mM L-glutamine and 1 mM Na-pyruvate
DMEM II	DMEM as above, but without Na-pyruvate
DMEM/Ham`s F12	DMEM and Ham`s F12 mixed 1:1 (Gibco-Invitrogen, no. 31330-038)
MEM-Earl`s	Minimal essential medium with Earl`s salts (Gibco-Invitrogen, no. 31095-029), supplemented with 2 mM L-glutamine and 20 mM HEPES
ECGM	Endothelial cell growth medium (PromoCell, basal medium no. C-22210, supplement pack no. C-39210), supplemented with 20 mM HEPES

Growth media:

RPMI, DMEM, DMEM II, DMEM/Ham`s F12 and MEM-Earl`s complete growth media were supplemented with 10 % heat inactivated fetal calf serum (FCS). Low-serum ECGM medium contained 2 % FCS.

Infection media:

Infection medium for *C. trachomatis* infection (RPMI for Hela cells, FT cells and FT tissue culture, DMEM for End1 cells) was supplemented with 5 % FCS.

Tab. 2.3: Cell culture buffers and reagents.

Name	Company
Phosphate buffered saline (PBS)	Gibco-invitrogen, no. 14190-169 (without Ca, Mg)
Trypsin/EDTA, 0.05 %	Gibco-Invitrogen, no. 25300-096, 0.05 % trypsin
Trypsin/EDTA, 0.25 %	Gibco-Invitrogen, no. 25200-056, 0.25 % trypsin
TripLE Express	Invitrogen, no. 12605-010
Trypsin inhibitor	Sigma, no. T6414
Accutase	Sigma, no. A6964
Collagenase	Biochrom, no. C2-28
HEPES	Gibco-Invitrogen, no. 15630080
L-glutamine	Invitrogen, no. 25030-024
Na-pyruvate	Sigma, no. 8636
Fetal calf serum (FCS)	Biochrom, no. S0115
HBSS (+Ca,Mg)	Gibco-Invitrogen, no. 14025-076
Gelatine	Sigma, no.G1393
Penicillin G	Sigma, no. PENNA-1MU
Streptomycin Sulfate	Sigma, no. S6501

2.1.5 Chemicals and reagents

If not stated otherwise, chemicals and reagents were bought from Roth, Sigma-Aldrich or Merck.

Tab. 2.4: Additional chemicals and reagents.

Chemical / reagent	Company
BSA	Biomol, no. 01400
DPX mountaint for histology	Fluka, no. 44581
DRAQ 5	Alexis, no. BOS-889-001-R200
Ganciclovir	Calbiochem, no. 345700
Hoechst	Sigma, B2261
Target retrieval solution	Dako, no. S1699
TritonX-100	Calbiochem, no. 648 466

2.1.6 Buffers and solutions

Tab. 2.5: Composition of buffers and solutions.

Buffer / solution	Composition
Blocking / permea-bilisation solution	0.1 % TritionX-100 and 0.2 % BSA in PBS (for staining of cells)
IFF blocking buffer	PBS + 1% BSA + 2% FCS, filter through 0.2 µm filter (for staining of tissue)
2x HBS	50mM HEPES 280mM NaCl 1,5mM Na2HPO4 adjust pH to 7.00 (± 0.05) with NaOH, exakt! Sterile filter (0.22 µm), store at 4 °C or -20 °C
Mowiol	2.4 g Mowiol 40-88 6 g Glycerin 6 ml H_2O; swell overnight 12 ml 0.2 M Tris pH 8,5 stir and heat to 60°C, centrifuge at 4000 min-1, aliquot
PBS	0.2 g KCl 0.2 g KH_2PO_4 8 g NaCl 2.16 g Na_2HPO_4 ad 1 l H_2O
PFA (para-form-aldehyde)	40 g PFA (for 8 % stock) dissolved by heating in warm 400 ml PBS (turbid solution), use NaOH to clarify ad 500 ml PBS, adjust pH to 7.5, stock aliquots -20ºC
SPG-buffer	75 g Sucrose 0.52 g KH_2PO_4 1.22 g Na_2HPO_4 0.72 g L-glutamic acid ad 1 l distilled H_2O, adjust to pH 7.4, sterile-filter, stored at 4°C
LB-medium	10 g Bacto-Trypton (Difco) 5 g yeast extracts (Difco) 10 g NaCl ad 1 l H2O, pH 7.5 100 µg ampicillin per ml media for bacterial selection

2.1.7 Antibodies

Tab. 2.6: Primary antibodies. Antibodies (including labelled primary antibodies) used for immunofluorescence staining of cells or tissue sections.

Antibody	Species	Company	Dilution
anti-acetylated tubulin	mouse	Sigma, no. T7451	1: 100 (tissue)
anti-ß-catenin	rabbit	Sigma, no. C2206	1: 50 (tissue)
anti-ß-catenin	rabbit	Abcam, no. 2365	1: 50 (tissue)
anti-*C. trachomatis*	goat	AbD Serotec, no. 1990-0804	1:25-1: 50 (tissue)
anti-*C. trachomatis* L2 MOMP	mouse	University of Washington, no. KK12	1:5000 (cells)
anti-cytokeratin-8	mouse	Santa Cruz, sc-8020	1:100 (cells) 1:100 (tissue)
anti-E-cadherin	rabbit	Cell Signaling, #3195	1:80 (cells)
anti E-cadherin	mouse	BD Transduction Laboratories, no. 610181	1:200 (tissue)
anti-EpCAM	mouse	Cell Signaling, no. 2929	1:80 (cells)
anti-chlamydial HtrA	rabbit	selfmade (Dr. Mirjana Kessler)	1:400 (cells)
anti-cleaved caspase 3	rabbit	Cell Signaling, no. 9664	1: 100 (tissue)
anti-LPS	rabbit	Milan, no. D183.1903	1:70 (cells) 1:150 (tissue)
anti-N-cadherin	rabbit	Abcam, ab18203	1:80 (cells)
anti-occludin	mouse	Invitrogen, no. 33-1500	1:100 (tissue)
anti-Oct4	mouse	Sigma, no. P0082	1:133 (cells)
anti-PECAM-1	mouse	R&D Systems, no. BBA7	1:50 (cells)
M30 CytoDeath (anti-cleaved cytokeratin-18)	mouse	Peviva, no. 10700	1:100 (cells) 1:100 (tissue)
FITC-anti-CD44	mouse	BD Pharmingen, no. 555478	1:150 (cells)
Alexa488-anti-Gfp	mouse	Santa Cruz, sc-9996	1:50 (cells)

Tab. 2.7: Secondary antibodies. Fluorescently labelled secondary antibodies used for immunofluorescence stainings. Dilution 1:200 for all applications.

Antibody	Company
Alexa 488- anti-mouse	Molecular Probes
Cy2-, Cy5- anti-mouse	Jackson Immuno Research
Cy3-, Cy5- anti-rabbit	Jackson Immuno Research
Cy2-, Cy3- anti-goat	Jackson Immuno Research

2.1.8 Primers

Tab. 2.8: Primers for RT-PCR. Primers used for oncogene detection via RT-PCR.

Target gene	Primer	Sequence
HTERT	forward	ACGGCGACATGGAGAACAA
	reverse	CACTGTCTTCCGCAAGTTCAC
SV40T	forward	CAGGCATAGAGTGTCTGC
	reverse	CAACAGCCTGTTGGCATATG
BMI1	forward	TTGGAGACCAGCAAGTATTGTC
	reverse	CGGGTGAGCTGCATAAAAATCT
GAPDH	forward	GGTATCGTGGAAGGACTCATGAC
	reverse	ATGCCAGTGAGCTTCCCGTTCAG

Tab. 2.9: Primers for qPCR virus titration. Primers bind to indicated positions in plasmid pHRgfpLOX; the binding region sequence is identical in plasmids pLOX-TERT-iresTK, pLOX-Ttag-iresTK, pLOX-CWBmi1 and pLOX-CW-CRE.

Target position	Primer	Sequence
position 1428-1446	forward	GGCTATTGAGGCGCAACAG
position 1576 - 1555	reverse	CAGTGGTGCAAATGAGTTTTCC

2.1.9 Plasmids

The plasmids pHRgfpLox, pLOX-TERT-iresTK, pLOX-Ttag-iresTK, pLOX-CWBmi1, pLOX-CW-CRE and psPAX2 were constructed by the group of Didier Trono (http://tronolab.epfl.ch/) and kindly provided by the group via Addgene (www.addgene.org, providing plasmid maps). The plasmid pCI-VSVG was a gift from Luk Van Parijs.

Tab. 2.10: Lentiviral plasmids. Names and descriptions of plasmids used to produce lentiviruses in this study.

Plasmid name	Gene product(s)	Description and reference
pHRgfpLox	eGFP	eGfp gene, CMV promoter, lox-site in 3' LTR; Addgene plasmid 12264, Salmon, et al. (2000), strain collection # H3600
pLOX-TERT-iresTK	hTERT, HSV-1 TK	hTERT and HSV-1 thymidine kinase (TK) genes, CMV promoter, lox-site in 3' LTR; Addgene plasmid 12245, Salmon, et al. (2000), strain collection # H3601
pLOX-Ttag-iresTK	SV40T, HSV-1 TK	SV40T and HSV-1 thymidine kinase (TK) genes, CMV promoter, lox-site in 3' LTR; Addgene plasmid 12246, Salmon, et al. (2000), strain collection # H3602
pLOX-CWBmi1	Bmi1	murine gene for Bmi1, CMV promoter, lox-site in 3' LTR; Addgene plasmid 12240, Salmon, et al. (2000), strain collection # H3603
pLOX-CW-CRE	Cre	nls-Cre gene (Cre-recombinase fused to nuclear localization signal), CMV promoter, lox-site in 3' LTR; Addgene plasmid 12238, Cudré-Mauroux, et al. (2003), strain collection # H3604
pCI-VSVG	VSV-G envelope	gene for viral envelope glycoprotein from Vesicular Stomatitis Virus; Addgene plasmid 1733, gift from Luk Van Parijs to the group, strain collection # L373
psPAX2	viral enzymes and structural proteins	genes for viral enzymes and additional structural proteins; Addgene plasmid 12260, Didier Trono laboratory, strain collection # L142

2.1.10 Equipment

The laboratory was equipped at modern laboratory standards.

Tab. 2.11: Additional equipment.

Equipment / material	Company
Phase contrast microscope	Olympus
Epifluorescence microscope	Leica DMR
Laser scanning confocal microscope TCS SP-1	Leica
Transmission electron microscope Leo 906E	Zeiss
ABIprism, RT-PCR cycler	Applied Biosystems
FACS Calibur	BD
cellZscope cell monitoring system	nanoAnalytics
filter inserts	BD Falcon
Cell culture flasks, dishes, multi-well plates	TPP
Cell culture 48-well plates	Corning

2.1.11 Kits

EndoFree Plasmid Midi / Maxi kits (Qiagen), for plasmid purification from bacteria stocks;

DNeasy Blood & Tissue Kit (Qiagen), for genomic DNA isolation;

RNeasy kit (Qiagen), for RNA isolation;

Quantitect SYBR Green RT-PCR kit (Qiagen), for quantitative RT-PCR and qPCR

2.1.12 Software and databases

ACT-1 vers.2 (fluorescence microscopy), Leica Confocal Software (confocal microscopy), Adobe Photoshop CS3 (image editing), FCS Express V3 (FACS analysis), SDS 2.2.0 (RT-PCR analysis), cellZscope (cell polarization monitoring), Microsoft PowerPoint (graphic design), Microsoft Word (text), Microsoft Excel (table calculation), NCBI homepage http://www.ncbi.nlm.nih.gov/ (literature database, BLAST sequence alignment), EndNote (bibliography management)

2.2 Cell culture and tissue preparation

2.2.1 Preparation of primary human fallopian tube (FT) cells

Primary human fallopian tube cells were either prepared via explants or digests.

The tissue was stored in a closed clean container, transported on ice to the lab within 2-3 h after surgery and rapidly processed. The tissue was placed in a sterile petri dish and continuously rinsed with PBS to remove all visible evidence of blood and to wash. Care was taken to remove connective tissue. For preparation of explants, the lumen of the tube was exposed by cutting it open longitudinally. The mucosal folds were dissected off by naked eye in a sterile environment, placed in a separate dish containing growth medium MEM-Earl`s + 10 % FCS + penicillin/streptomycin (Pen/Strep) and further cut into small pieces. The pieces were placed in 48well, 24well or 12well dishes in growth medium with 10 % or 40 % FCS supplemented with Pen/Strep. Small volumes of medium were used to

ensure tissue pieces could attach to well bottom surfaces. Samples were incubated in a humidified atmosphere at 5 % CO_2 and 37 °C. On the next day or when most pieces had adhered, medium was filled up to the standard volume used per well. Latest 3 days after preparation all cultures were kept in normal growth medium (10 % FCS) for usually 1-2 weeks until immortalization or subculture. Explants were removed after 3-4 days, or latest when outgrown directly surrounding cells were near confluent. This method was adapted from Saridogan, et al. (1997) Human Repr., 12 (7), 1500-1507.

When cells were prepared using enzymatic digest, tissue was transported and washed as described above. Then the fallopian tube was perfused with 0.5 % collagenase in HBSS for 10 min. After incubation, the mucosal folds were cut off, placed in an Eppendorf reaction tube and incubated in 0.25 % trypsin/EDTA at 37 °C for 30 min while continuously shaking. The samples were centrifuged at 50 g (in an Eppendorf centrifuge) for 3 min. The pelleted cells were resuspended in growth medium with Pen/Strep and seeded into multi-well plates. This method was adapted from Ando, et al. (2000) Human Repr., 15 (7), 1597-1603.

In another protocol using enzymatic digest the following procedure was performed. The tissue was transported and washed as described above. After removal of surrounding tissue, the fallopian tube was minced extensively using a scalpel. Minced tissue was placed in a 15 ml Falcon tube and incubated in 5 ml pure TripLE Express enzyme at 37 °C in a water bath for 30 min. Afterwards the supernatant containing loosened cells was collected without disturbing the rest of the tissue. 7 ml DMEM/Ham`s F12 + 10 % FCS + Pen/Strep was added. Cells were pelleted by centrifugation with 400g (ca. 1500 rpm in a Falcon tube centrifuge) for 5 min. Supernatant was discarded. The cells were resuspended in fresh media and seeded in 25 cm² flask. This method was described by Jazedje, et al. (2009) J Transl Med., 7(46) doi:10.1186/1479-5876-7-46.

2.2.2 Preparation of primary human FT tissue for tissue culture

The tissue was stored in a closed clean container, transported to the lab and rapidly processed. The tissue was placed in a sterile petri dish and continuously rinsed with PBS to remove all visible evidence of blood and to wash, at least twice. Care was taken to remove connective tissue. Then tissue pieces of about 0.5 cm length were obtained by transverse sections and placed into 4-6 cm dishes in growth medium without antibiotics, as pieces were usually used for infection studies.

2.2.3 Preparation of primary human umbilical vein endothelial cells (HUVEC)

The umbilical cord was placed in a large sterile Petri dish. A metal buttoned cannula was inserted into the vein and fixed with a clamp. The vein was rinsed two times with pre-warmed 37 °C HBSS. 10 ml pre-warmed 0.5 % collagenase in HBSS were used to remove remnants of HBSS and after clamping of the other end used to fill the vein completely. The cord was incubated 15 – 30 min at 37 °C in the Petri dish rapped in a sterile plastic bag in a humidified incubator with 5 % CO_2. After incubation, the lower clamp was taken off, the collagenase solution collected in a petri dish and the vein was rinsed two times with 20 ml HBSS each. Wash flow through was collected together with collagenase solution in a 50 ml Falcon tube and centrifuged 5 min with 1200 rpm. Cells were resuspended in growth medium ECBM containing Pen/Strep and seeded into a 75 cm² flask coated with 0.5 % gelatine. The cells were incubated in a humidified incubator with 5 % CO_2 and 37 °C. After 1 h, cells were rinsed two times with HBSS and fresh growth medium containing antibiotics was added.

2.2.4 Cell culture propagation

Cell culture work was performed under sterile conditions. Cells were routinely grown in growth medium (see chapters 2.1.2 and 2.1.4) in culture flasks in a humidified incubator at 5 % CO_2 and 37 °C. Culture vessels for HUVEC cells were coated with 0.5 % gelatine in PBS for 1h at RT and then washed with PBS. Cells were splitted before reaching complete confluence. Cells were washed with RT PBS and then 1 ml trypsin pre-warmed at 37 °C (for Hela, End1, HFF-1 and HUVEC cells) or accutase (for FT cells) was added per 75 cm² flask. Cells were incubated in the humidified incubator until the cells detached (at RT for HUVEC). Growth medium with 10 % FCS was added to neutralize trypsin activity. In case of HUVEC cells grown in low-serum medium, 1 ml trypsin-inhibitor was added directly to detached cells, PBS was added to wash and the cells were pelleted for 5 min at 800-1000 rpm in a Falcon tube centrifuge. Pellets were resuspended in growth medium. Diluted cells were splitted into new flasks for further culture and incubated as described above.

The volumes typically used were: 12 ml medium per 75 cm² flask, 6-7 ml per 25 cm² flask, 2 ml per sixwell, 1 ml per 12well, 0.5 ml per 24 well and 0.25 – 0.5 ml per 48 well.

2.2.5 Isolation of cell clones

In order to obtain single cells for clonal expansion, mixed cell cultures were treated with cloning rings or subjected to FACS single cell sorting. Cloning rings were fixed with sterile melted agarose around single colonies in cultures with very low confluence. Cells within rings were treated with trypsin as used in cell culture propagation and transferred to a

new culture well. For FACS single cell sorting, samples were prepared as described for determination of virus titer and infection rate via FACS (see 2.3.3). In addition, cells were stained with the red fluorescent membrane dye FM 4-64 (Invitrogen) at 0.1 µl per 1 ml media. Cell sorting was performed by co-workers of the flow cytometry core facility at the institute.

2.3 Working with lentiviruses

2.3.1 Notes on the method of reversible immortalization

A scheme sumarizing the strategy for reversible immortalization is displayed in Fig. 3.4 and a summary of the method is provided here. For details of lentiviral plasmids see above (chapter 2.1.9), for lentiviral integration please refer to the introduction.

For production of recombinant lentivector (LV) particles, three plasmids are co-transfected into producer 293T cells. The plasmids encode either the target protein (oncoprotein hTERT, SV40T or Bmi1; or recombinase Cre), the viral envelope protein (Vesicular Stomatitis Virus Glycoprotein, VSV-G) or the viral structural proteins and enzymes. Produced LV particles are released into the cell supernatant. Recombinant viruses are harvested with the cell supernatant. For immortalization, primary cells are infected with recombinant LV particles carrying the oncogenes. The target gene sequences integrate into the genome of the infected cells and are flanked by lox-sites after integration. Excision of oncogenes is mediated by the recombinase Cre and designated as re-mortalization. To achieve excision, immortalized cells are superinfected with recombinant LV particles carrying a self-excisable Cre recombinase construct. The gene integrates into the genome as well. Expressed Cre recombinase recognizes the lox-sites and excises the integrated oncogenes. As the gene encoding the recombinase itself is flanked by lox-sites, too, also this sequence is excised after sufficient production of recombinase. To select re-mortalized cells, the viral thymidine kinase (TK) suicide gene is encoded on the hTERT and SV40T immortalizing plasmids in addition to the oncogen for negative selection of immortalized cells.

2.3.2 Generation of recombinant lentivector particles

Lentiviruses were produced in 293T cells via calciumchloride transfection of lentiviral plasmids. $2x10^6$ 293T cells were seeded in a 10cm dish in 10ml DMEM + 10 % FCS + 2mM L-glutamine without antibiotics. On the next day, the cells were simultaneously transfected with 20 µg target plasmid (coding for Gfp, an oncoprotein, or Cre recombinase), 6 µg envelope plasmid (coding for VSV-G) and 15 µg packaging plasmid (plasmid psPAX2). The DNA was diluted in H_2O ad 250 µl, and 250µl $CaCl_2$ (1M, RT) was added. During continous vortexing, the DNA/$CaCl_2$ mixture was added slowly dropwise to

500µl 2x HBS (RT) and incubated for 30 min at RT. 1 ml transfection solution was pipetted dropwise on the cells in one dish. The dish was gently agitated to mix. On the next morning, cells were washed with warm PBS (37 °C) and 10 ml fresh medium were added carefully. One day later, the supernatant (i.e. medium containing lentivector particles) was collected, and 8 ml fresh medium was added to the cells. The supernatant was centrifuged at 2000 rpm 4°C for 7min to remove cell debris and stored at 4 °C over night. On the next day, the supernatant was collected from cells, centrifuged at 2000 rpm 4°C for 7min and clear supernatants from day1 and day2 were pooled. The solution containing lentiviruses was filtered through 0.45 µm pore filter. Small aliquots were stored at -80°C.

2.3.3 Determination of virus titer / infection rate via FACS

This procedure was applied to titrate viruses encoding Gfp. Hela cells ($1.5x10^5$) were seeded in six-well plates in 2 ml medium per well. On the next day, medium was changed in the afternoon and 1 ml fresh medium was added per well. The polybrene stock (Sigma, 10 mg/ml) was diluted with PBS to 1 mg/ml working solution, of which 1ml was added per ml medium. Generally, six samples were prepared per titration assay: cells untreated, treated only with polybrene, or infected with 1 µl, 10 µl, 100 µl or 1000 µl of virus containing supernatant. On the next morning, the medium was removed, cells were washed with pre-warmed PBS (37 °C) and 2 ml fresh medium was added per six-well. Cells were harvested three or four days post infection, harvested with trypsin as usual, washed with PBS and centrifuged (1000 rpm in Falcon, 5-7 min). Pelleted cells were resuspended in and fixed with 2 % PFA for 20 min at RT. After PBS wash and addition of fresh PBS (approx. 500 µl), cells were analyzed. When cells were in culture for at least 4 days post infection, there was no free virus and cells could be analyzed without fixation. For analysis of living cells, trypsinized cells were resuspended in 500 µl 4 % FCS in PBS. In case of living cells, propidium iodid was added to discriminate dead cells. Propidium iodide stains DNA in dead cells due to their permeable cell membrane. Samples were vortexed very shortly.

Cells were analyzed in a FACS Calibur (BD) for Gfp expression in channels FL-1 versus FL-2. Data were analyzed using the software FCS Express and the percentage of Gfp expressing (i.e. infected) cells was measured by placing a gate discriminating between Gfp-negative and Gfp-positive cells. Untreated non-infected cells were used as negative control. The histograms display cells after exclusion of cell debris by size and of dead cells by propidium iodide staining.

In a typical titration experiment, only dilutions yielding to 2-20% GFP positive cells were considered for titer calculations (below 2%: chance of unreliable results, above 20%:

chance for each positive target cell to be transduced twice significantly increases, resulting in underestimation of the number of transducing particles).
The virus titer in cell transducing units (ctu) was calculated using the following formula:
ctu / ml = number of target cells x (% of infected cells/100) / volume of supernatant (in ml)

2.3.4 Determination of virus titer via quantitative PCR

In order to assure functionality and determine efficiency of the LV packaging system, the obtained LV particles were titrated by quantitative real time PCR (qPCR) in comparison to LV particles encoding Gfp instead of an oncoprotein in the same plasmid background. In qPCR, the number of lentivector constructs stably integrated into the target cells is measured. The Gfp-encoding LV particles were also titrated via FACS analysis (see above), the standard titration protocol in the group, to obtain comparable values.
A standard curve was generated for absolute quantification of viral integration. For this purpose, Hela cells were infected with the Gfp-encoding lentivector particles used in this study to obtain cells with a defined number of integrations (ideally 1). Cells were analyzed in FACS analysis (as described above) and cells were sorted in the core facility flow cytometry in the institute. Gfp-positive Hela cells were obtained that carried in average 0.9 viral integrations per cell. Genomic DNA was prepared (see chapter 2.7.2) from these cells and used for quantitative PCR (see chapter 2.7.5) with primers amplifying a part of the gag-gene/packaging signal present on all target gene-plasmids of this system. The standard curve was calculated with Excel software displaying copy number versus delta Ct-value obtained in qPCR.
For titration, Hela cells were infected with the lentivector particles as described above. Genomic DNA was prepared and qPCR was performed (see below). The standard curve was used to calculate integration numbers from delta Ct-values obtained in qPCR.
The titer in cell transducing units (ctu) was calculated using the following formula:
ctu / ml = number of target cells x number of copy per cell of the sample / volume of supernatant (in ml)
As qPCR titers were higher than FACS titres, both titres were compared for the Gfp-encoding lentivector particles and the multiplicity factor was determined, by which both titers differed. For comparibility, titers of all other lentivector particles were calculated accordingly to match the Gfp-construct FACS titer.

2.3.5 Infection with lentivirus (immortalization, re-mortalization)

Cells were seeded the day before infection in the evening or primary isolates were used on the day of infection. Directly before infecting cells, the medium was exchanged and fresh medium was added (half the volume used for normal culture: 1 ml per six-well, 500

µl per 12-well, 250 µl per 24-well). 10 µl polybrene (1 mg/ml diluted in PBS) was added per ml medium (final concentration 10µg/ml). Virus containing supernatant was added according to multiplicity of infection (MOI). On the next morning, medium was removed, cells were washed once with pre-warmed PBS (37 °C), and fresh medium was added (2 ml per six-well, accordingly for smaller wells) and cells were further incubated.

For oncogene excision (re-mortalization), cells were infected with lentivector particles encoding the recombinase Cre. Various MOIs were tested with TMNK-1 control cells. For negative selection of cells with non-excised constructs, cells were treated with 1 µM ganciclovir starting 3 days post infection for 10 days (as used by Salmon, et al., 2000). Ganciclovir is a nucleoside analogon phosphorylated by the viral HSV-1 TK. The resulting nucleotides are toxic as their incorporation during DNA replication leads to a block thereof and eventually to cell death (Salmon, et al., 2000; Caruso, 1996).

During infection with Cre-encoding virus (re-mortalization) of TMNK-1, cells were sub-cultured during the course of the experiment as follows: sample MOI1 three times, sample MOI5 twice, non-infected sample four times.

The HUVEC samples were analyzed with SYTOX dead cell stain (Invitrogen) at the end of the Cre experiment following manufacturer's protocol, with analysis in a spectrophotometer plate reader. Living cells are impermeable to this nucleic acid stain, which therefore specifically stains dead cells.

2.4 Working with *Chlamydia*

2.4.1 *Chlamydia trachomatis* stock preparation

Hela cells were cultivated in a 75 cm² flask and infected with *C. trachomatis* at approx. MOI2 (serovar L2) or 50 µl aliquot from old stock (serovar D) by putting the bacteria into infection medium on the cells. Two hours post infection, medium was exchanged. Cells were incubated for 48h for serovar L2, 72h for serovar D in a humidified cell incubator at 35 °C. Cells were then scraped into the culture medium, transferred into 50 ml Falcon tube filled with 7.5 ml sterile glass beads (1-1.5 mm^2) and vortexed for 3 min (2500 rpm). The chlamydial suspension was transferred to a new tube and used to inoculated Hela cells in 10x 150 cm² flasks with each 200 µl directly into the old medium. The medium was exchanged two hours post infection for infection medium. Cells were incubated for 48h for serovar L2, 72h for serovar D in a humidified cell incubator at 35 °C. Cycloheximid was added, if cells tended to overgrow. To harvest, medium was reduced to 10 ml in each flask. Cells were scraped into the medium, transferred into 50 ml Falcon tubes filled with 7.5 ml sterile glass beads (1-1.5 mm^2), max. 30 ml per tube, and vortexed for 3 min (2500 rpm). The chlamydial suspension was transferred to new Falcon tubes, and centrifuged at 2000 rpm for 5 min at 4°C in a Haermle centrifuge. The supernatant

was transferred to SS34 tubes and centrifuged at 18.000x g in the Sorval centrifuge at 4°C for 30 min. The chlamydial pellet was resuspended in 10 ml SPG buffer, transferred to a Falcon tube, and resuspended in a syringe 5x with G20 needle and 3x with a smaller G23/26 needle. 50 µl aliquots were stored at -80 °C.

2.4.2 Determination of *C. trachomatis* stock titre

Hela cells were seeded in DMEM with 10 % FCS, L-glutamine and Na-pyruvat, thereby cells from a 75 cm² flask were distributed 1:4 to a complete 24-well plate. On the next day, cells should be 80-90 % confluent, not more. *C. trachomatis* was diluted in infection medium containing only 5 % FCS as follows: 1:500, 1:1000, 1:2000, 1:5000, 1:10.000, 1:20.000; infections were performed in duplicates (two wells per dilution), with each 250 µl. Medium was exchanged two hours post infection. Samples were incubated for 24h in a humidified cell incubator at 35 °C.

On the next day, chlamydial inclusions were counted under the phase contrast microscope using the 40x objective. Alternatively, the Hela cells were seeded on coverslips in 12wells in the beginning, used for immunofluorescence staining for *Chlamydia* and inclusions were counted from pictures acquired with the epifluorescence mircroscope. For the latter procedure, the medium was removed, the cells fixed with 350 – 500 µl ice cold methanol per well for 10 min at RT and wash twice with 1xPBS. For uniform staining of chlamydial inclusions it was advisable to store the plate in 70 % ethanol 500 µl per well at 4°C overnight. Sampled were blocked with 300 µl of 0.2 % BSA in 1x PBS for 30 min at RT. Blocking solution was removed and 300 µl primary antibody dilution was added per well (anti-MOMP for L2) in 0,2% BSA in 1xPBS. Samples were incubated for one hour at RT and washed twice with 1xPBS. Hoechst was diluted 1:10.000 in 0,2% BSA in 1xPBS and the secondary antibody was added 1:100. 300 µl of the solution was added per well. The plate was incubated for one hour in the dark, and then washed twice with 1xPBS. 500 µl fresh 1xPBS was added per well and the coverslips removed and mounted on object slides in Mowiol. Slides were stored at 4 °C.

The samples were counted from the dilution, at which 30 – 60 inclusions were visible per optic field. Per well 10 random optic fields were counted, with 5 crosswise and 5 lengthwise. Duplicates gave 20 values and the average was used for calculations. The titer was calculated as follows, expressed in inclusion forming units (ifu):

ifu / ml = Ø * 2975,2 * dilution factor * 4

2.4.3 Infection of cells with *C. trachomatis*

Infection was generally performed in half the amount of medium used for cell culture propagation, and with infection medium containing only 5 % FCS. Chlamydial stock

solution was freshly thawed from -80 °C. Cells were infected by directly adding chlamydiae to the infection medium. Samples were incubated in a humidified cell incubator at 35 °C.

2.4.4 Infection of tissue with *C. trachomatis*

Tissue samples were infected on the day of dissection with *C. trachomatis* serovar D or L2 with 5*10^6 ifu in RPMI medium + 5% FCS and incubated at 35°C 5% CO_2 in a humidified incubator. To detect bacterial progeny, which was able to re-infect cells, supernatants were collected after usual incubation time and applied to Hela cells. Cell samples were infected as described above.

2.5 Cell biological methods

2.5.1 Immunofluorescence staining of cells

Medium was removed from cells grown on coverslips, cells were washed once carefully with PBS, and fixed in 2 – 4 % PFA for 20 – 30 min at RT. Samples were rinsed two times with PBS and either stored in fresh PBS at 4 °C until staining or used fresh. After additional 10 min wash, samples were blocked and permeabilised in 0.2 % BSA in PBT (PBS + 0.1 % Triton X-100) for 30 min in the dark. The first antibody was diluted in 0.2 % BSA in PBS or PBT and ca. 30 µl per sample were incubated for 1 – 1.5 h at RT in a wet chamber to prevent samples from drying. Cells were washed three times 5 min with PBS on a shaker. The fluorescently labelled secondary antibody and a DNA staining dye (Hoechst for epifluorescence microscopy or Draq5 for confocal microscopy, both diluted ca. 1:1000) were diluted in 0.2 % BSA in PBS or PBT and ca. 30 µl per sample were incubated for 1 h at RT in a wet chamber in the dark. From here on, samples were kept in the dark. Cells were washed three times 10 min with PBS on a shaker, rinsed once shortly in distilled water to remove salt crystals and mounted in Mowiol on object slides. Dried samples were stored at 4 °C. Images were acquired with an epifluorescence microscope using ACT-1 software.

2.5.2 Polarization of cells

$4*10^4$ cells per insert were seeded on PET membrane filter inserts (BD Falcon® 353103, 1.0 µm pore size) for 12-wells. Cells were grown for two weeks in MEM-Earl's growth medium with penicillin and streptomycin. In continuous simultaneous measurements of all wells, trans-epithelial electrical resistance (TEER) was determined with the automated computer-controlled cell monitoring system cellZscope (nanoAnalytics, Germany) using the cellZscope software (nanoAnalytics). In each well, TEER was measured sequentially once per hour.

2.5.3 Transmission electron microscopy

For fine structural analysis, cell-layers on filters were fixed with 2.5% glutaraldehyde, postfixed with 0.5% osmium-tetroxide, contrasted with 0.1% tannic acid (all from Polysciences) and with 2% uranyl-acetate (Serva), dehydrated in a graded ascending ethanol series, and embedded in Polybed (Polysciences). Filters were first polymerized with a thin film of resin, cut to size, stacked and re-embedded in resin. After polymerization, specimens were cut at 60 nm and contrasted with 3% lead citrate (Merck, prepared after Reynolds, 1963). Specimens were analyzed in a Leo 906E transmission electron microscope (Zeiss, Oberkochen, Germany) equipped with a sidemounted digital camera (Morada, SIS-Olympus, Münster, Germany). After fixation with glutaraldehyde, the sample preparation and transmission electron microscopy were performed by the co-workers of the microscopy core facility at the institute.

2.5.4 Karyotype analysis

Karyotype analysis was performed in cooperation by Dr. Marc Trimborn from the Institute for Medical Genetics, Campus Virchow-Klinikum, at Charité University Hospital, Berlin, Germany. For chromosome preparation, colcemid was added to cell cultures 1h prior to the preparation. Cells were trypsinized, centrifuged, and the pellet resuspended. Samples were filled up with 5 ml of prewarmed 0.4 % KCl solution (37°C) (hypotonic salt solution) and incubated for 10 min at 37 °C in a water bath. Samples were centrifuged, approx. 4 ml of the supernatant discarded and the pellet resuspended in the residual volume. The cells were fixed with 1 ml ice-cold fixative (methanol/acetic acid 3:1) dropwise and slowly, resuspended carefully and another 4 ml were added. Samples were centrifuged, resuspended in 5 ml fresh ice-cold fixative, and this step was repeated once. Most of the supernatant was discarded and samples resuspended. For slide preparation, glass slides were cleaned with ethanol. One or two drops of the suspension were dropped on a slide and let to disperse. Slides were dried under a lamp in wet atmosphere above water bath. Chromosomes need to be free from cytoplasm.

Samples of chromosome spreads were incubated with trypsin followed by staining with Giemsa-solution, leading to GTG-banding of chromosomes. For banding, slides were incubated over night at 60 °C. On the next day, 2.5 ml trypsin (2.5%) were thawed and diluted with 22.5 ml 0.9% NaCl to the final concentration 0.25%. Samples were trypsinized at RT for 20s to minutes, optimized intervals needed to be determined for every preparation. Slides were carefully rinsed with tap water. For staining, slides were incubated for approx. 14 min with 5 % Giemsa in phospate buffer (pH 6,88); again, optimized incubation times were determined daily. Staining was checked under the

microscope and prolonged if necessary. Pictures were acquired and chromosomes ordered according to their consecutive numbers to obtain karyograms.

2.6 Histological methods

2.6.1 Paraffin embedding and microtome sections

Tissue was washed with PBS, fixed in 4 % PFA for 16-24h, washed twice with PBS and stored in PBS at 4 °C until embedding.

Tissue was dehydrated via alcohol series and perfused with paraffin in a Shandon Citadell 1000 rondell, with the following steps, each 1h incubation: 60 % ethanol, 70 % ethanol, 80 % ethanol, 90 % ethanol, 2x 96 % ethanol, 2x isopropanol, 2x acetone, 2x paraffin for each 2h. Tissue stayed in liquid paraffin until embedding. Tissue was embedded in paraffin blocks using a Microm Paraffin Console and cut with a Microm Paraffin Rotation Microtome. Prepared sections on object slides were incubated over night at 37 °C before use.

2.6.2 Hematoxillin-eosin staining

For deparaffinisation, paraffin samples were incubated two times 15 min in xylol, two times 5 min in absolute ethanol, 3 min in 90 % ethanol, 3 min in 70 % ethanol, 5 min in 50 % ethanol and 5 min in bidest water. Samples were incubated in hematoxillin for 5 min, then rinsed under streaming water for 10 min, and in 0.5 % eosin for 2 min. Slides were quickly washed in bidest water and 70 % ethanol, then incubated in 90 % ethanol for 3 min, two times in absolute ethanol for each 5 min, and two times in xylol for each 15 min. The samples were covered with DPX mountaint for histology.

2.6.3 Immunofluorescence staining of formalin-fixed paraffin-embedded (FFPE) tissue

Before dewaxing and antigen retrieval of paraffin tissue sections Dako target retrieval solution was heated to 95 °C in a glass container in a water bath. Sample slides were placed in a glass slide holder, incubated two times 5 – 10 min in xylene with agitation at regular intervals, 2x (10 – 20 sec in 100 % ethanol), 10 – 20 sec in 90 % ethanol, 10 – 20 sec in 70 % ethanol, 10 – 20 sec in 50 % ethanol followed by two washes in water. Then the slide holder was transferred into pre-heated target retrieval solution and incubated for 30 min in the water bath, then 20 min at RT and 5 min under running water.

For immunofluorescence labelling, the slides were removed from running water and wiped around the section. 100 µl PBS was added on the section to prevent it from drying out. An ImmEdge pen was used to make rings around the sections. The slide was shaken to remove excess PBS and 100 µl of blocking solution / IFF = immunofluorescence buffer

(PBS + 1% BSA + 2% FCS, filtered through 0.2 µm filter) was added on the section. The slides were placed in a moist chamber to prevent drying. All following steps were carried out at RT, the volumes were 20 – 40 µl depending on the size of the sections. The first antibody diluted in IFF was incubated 90 min. After washing three times 5 min with PBS, the fluorescently labelled secondary antibodies (1:200) and Draq5 (1:800) were diluted in IFF and incubated for 60 min in the dark. Samples were washed three times 5 min with PBS and 1x with destilled water. For mounting, Mowiol was dropped on the sections on object slides and cover slips were put above. Dried samples were stored at 4 °C in the dark. This method was described by Robertson, et al. (2008), BMC Cell Biology, 9:13. Confocal images were acquired with Leica TCS SP-1 microscope using Leica Confocal Software.

2.7 Working with nucleic acids

2.7.1 DNA plasmid purification

Plasmids were obtained each transfected in *Escherichia coli* laboratory strain Stbl3 and purified from bacterial cultures grown in LB-medium + ampicillin at 37 °C. The EndoFree Plasmid Midi / Maxi kits (Qiagen) were used following the manufacturer's protocol to purify plasmids. DNA concentrations were determined with a spectrophotometer (ND-1000, Nanodrop, Wilmington, USA).

2.7.2 Genomic DNA purification

DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. The DNA was eluted in prewarmed (56 °C) ultra-pure water.

2.7.3 RNA purification

RNA was isolated using RNeasy kit (Qiagen) following the manufacturer's protocol using the spin technology. Approx. $1*10^6$ cells were pelleted by centrifucation, resuspended in 300-350 µl lysis buffer RLT and used directly or stored at -20°C. Cells were homogenised using a QiaShredder-column and RNA was purified using the RNeasy spin column as described in the protocol. RNA concentrations were determined with a spectrophotometer (ND-1000, Nanodrop, Wilmington, USA).

2.7.4 Real time RT-PCR

Real time RT-PCR was performed using Quantitect SYBR Green RT-PCR kit (Qiagen) in a one step reaction combining reverse transcription (RT) and polymerase chain reaction (PCR) on cDNA. Reactions performed in 25 µl contained 100 ng RNA (in 10 µl), 12.5 µl SYBR Green mix, 0.25 µl RT-mix and 0.4 µM per primer and nuclease-free water ad 25

µl. Reactions were analyzed in 96-well plates in an ABIprism cycler (Applied Biosystems). The following program was used: 30 min 50°C, 15 min 95 °C, 45 cycles of 20 sec 94°C/40 sec 60°C/40 sec 72°C, then melting curve determination with 15 sec 95 °C, 15 sec 60 °C, 15 sec 95 °C. Reactions for *GAPDH* were performed for every RNA sample and used as internal controls. Analysis was performed using SDS 2.2.2 software and graphs were generated using Excel table calculation software.

2.7.5 Quantitative PCR

Quantitative PCR (qPCR) on genomic DNA (to determine viral titers, see above) was performed without reverse transcription (RT-mix substituted with nuclease-free water) and using 5 ng and 50 ng DNA per reaction (for titration standard curve) or 35-160 ng DNA (for titration); otherwise, the same protocol as above was followed.

3 Results

The results of this study are divided topically into two parts. The first part addresses immortalization of human primary cells, with focus on cells from fallopian tubes, a natural site of infection for *Chlamydia* (part 3.1). In the second part, the chlamydial infection is analyzed in a newly established human fallopian tube *ex vivo* tissue culture to gain insight into the host-pathogen-interactions in an "*in vivo*-like" situation (part 3.2).

3.1 Part 1: Immortalization of primary human cells to generate a new infection model

Cancer cell lines and immortalized cells are well established model systems to study the processes of pathogen-host-interaction during infection. However, these cells have indefinite life span and lack contact inhibition. Therefore, they differ substantially from the pathogen's primary host cells. In the case of cancer cell lines, which may have been cultivated in laboratories for decades, the changes resulting from transformation are usually unknown, especially the number of affected genes and their impact. However, primary cells, which better mimic the natural *in vivo* situation of host cells, often are difficult to acquire and enter senescence rapidly. This complicates the direct and routine work with primary cells.

Therefore, we aimed to establish new infection models by using primary human endothelial and epithelial cells for reversible immortalization. This process would allow the generation of a stable stock supply and the possibility to revert cells later back to a "primary-like" phenotype.

3.1.1 Isolation and cultivation of primary human epithelial and endothelial cells

As a first step, primary human fallopian tube epithelial cells and endothelial cells from the human umbilical cord had to be isolated directly from primary tissue. Therefore, establishment of the preparation of fallopian tube tissue and the isolation and culture of fallopian tube cells were a major part of this work.

Healthy human fallopian tubes were obtained from the Clinics of Obstetrics and Gynecology, Campus Virchow-Klinikum and Charité Mitte, at Charité University Hospital, Berlin, Germany, when tissues were removed for medical reasons. Material transfer and scientific usage were approved by the ethics commission of the Charité University Hospital and were carried out with given consent of patients. Varying tissue parts were obtained, ranging from the beginning of the tube next to the ovary (infundibulum with fimbrial segment) to the end near the uterus (isthmic segment). In each case, it depended on the performed surgery which tissue part was obtained. Tissues were usually dissected

within 2-3 h after surgery. The surrounding tissue was removed carefully to obtain the free tube (Fig. 3.1A). Afterwards, the fallopian tube was opened longitudinally to expose the inner mucosal folds (Fig. 3.1B). The mucosal folds line the lumen of the tube, appear brownish-yellowish and contain the single-layer simple epithelium.

For the preparation of primary fallopian tube (FT) cells, different isolation strategies were tested. The most effective approach was carefully taking small tissue explants of the inner mucosal folds into culture. Primary cells grew from the rim of the tissue into the medium, where explants had contact with the bottom of culture vessels (Fig. 3.1C). Cells which were loosened during the isolation process adhered to the vessels and formed colonies of primary cells (Fig. 3.1D). In a second approach, cells were isolated by mechanical shearing and subsequent enzymatic digestion of small tissue pieces. Coating of culture vessels with collagen or gelatine did not improve the outcome of both strategies. A third protocol for the isolation of cells from FT tissue combined mincing the whole tube with subsequent digestion. The protocol was introduced only recently into this work and yielded mixed cultures of primary cells.

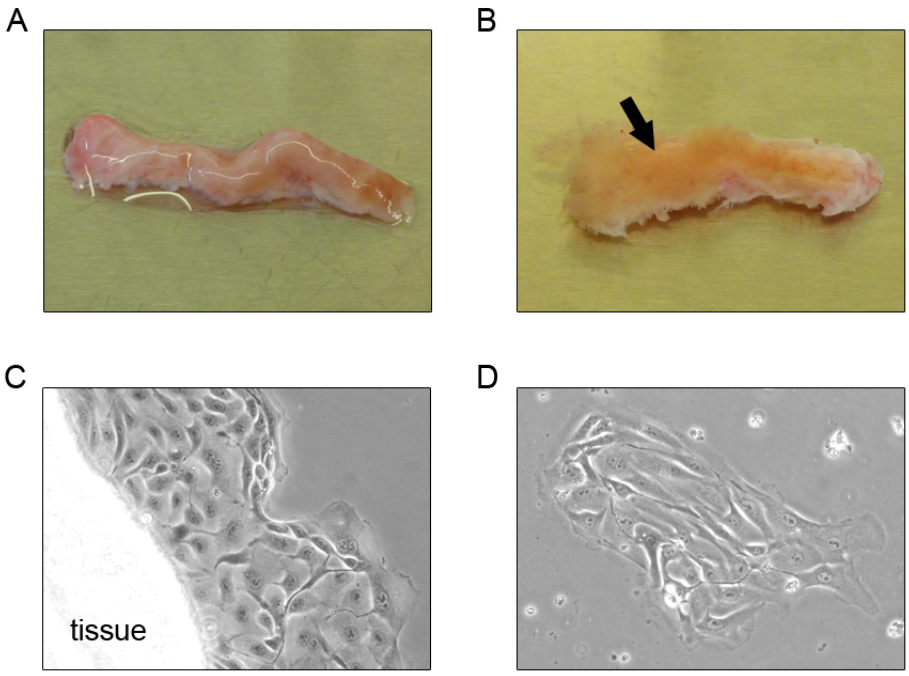


Fig. 3.1: Fallopian tube tissue and cell preparation. A) Four cm long part of a human fallopian tube (FT) after removal of surrounding tissue. **B)** Tissue was opened longitudinally to expose the lumen with inner mucosal folds, which appeared brownish-yellowish (arrow) and contained the simple columnar epithelium. **C)** Primary cells growing out of a FT tissue explant (tissue, appearing white) four days after preparation. **D)** Isolated cell island of FT primary cells four days after preparation.

The obtained primary FT cell populations were investigated for the presence of epithelial cells within the isolates. EpCAM (epithelial cell adhesion molecule) is an adhesion marker and ubiquitously expressed in differentiated epithelia within the genital tract. EpCAM was expressed throughout the cytoplasm in an isolated primary epithelial cell within a mixed primary cell population (Fig. 3.2 top). In End1 control cells, an epithelial cell line, EpCAM localized at cell contacts. Cytokeratin-8 (CK-8) is a cytoskeleton component present in all epithelial cells in simple epithelium, including FT epithelium. A fraction of primary FT cells was positive for CK-8, which was localized throughout the cytoplasm comparable to End1 cells (Fig. 3.2 bottom). This analysis confirmed the presence of epithelial cells in the isolated primary FT cell populations. The precondition for immortalization is the isolation and culture of primary cells. This task was successfully completed, as epithelial cells were detected in mixed populations.

In contrast to fallopian tube cells, a well-functioning protocol was available for the isolation and separation of primary endothelial cells from the human umbilical vein from surrounding tissue. Nearly pure cell populations could be obtained with this protocol.

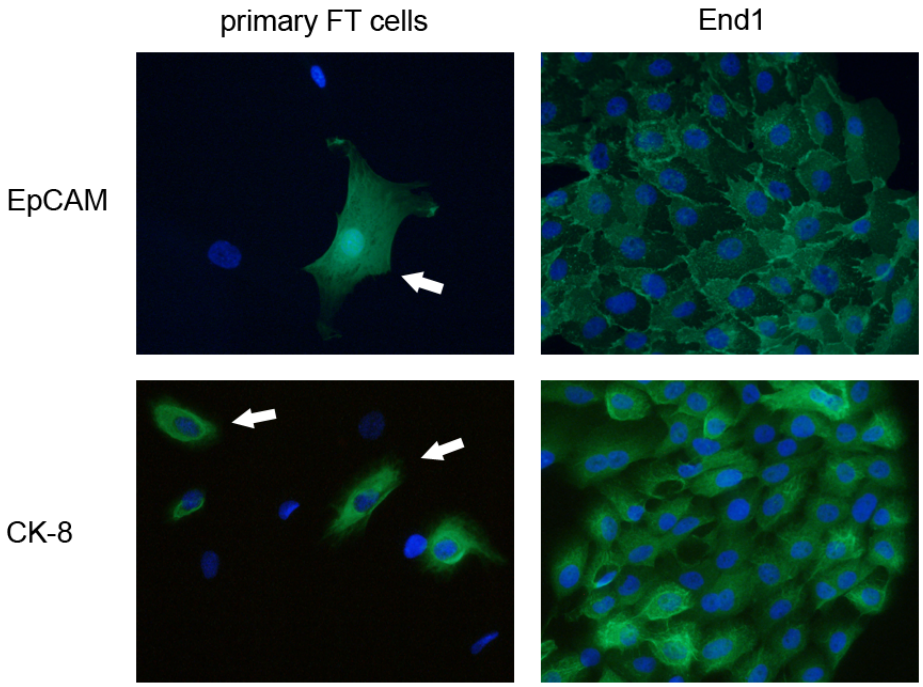


Fig. 3.2: Primary fallopian tube cell populations contain cells of epithelial origin expressing EpCAM and cytokeratin-8. Immunofluorescence analysis of isolated primary fallopian tube (FT) cell populations and End1 epithelial cell line for epithelial markers EpCAM (top) and cytokeratin-8 (CK-8, bottom). Top: EpCAM (green) was detected in an isolated primary epithelial cell (left, arrow) and at cell contacts in confluent End1 cells (right). Bottom: Cytokeratin-8 (CK-8; green) was localized in the cytoplasm in both primary epithelial cells (left, arrows mark examples) and End1 cells (right). DNA was stained with Hoechst (blue).

Therefore, human umbilical vein endothelial cells (HUVEC) were used to establish single steps of the complex process of reversible immortalization. For preparation of primary endothelial cells, human umbilical cords were obtained shortly after childbirth from clinics of Obstetrics and Gynecology, Campus Virchow-Klinikum and Campus Charité Mitte, at Charité University Hospital, Berlin, Germany. After removal of blood from the vein, endothelial cells were isolated by enzymatic digestion. Flow through after incubation contained the endothelial cells.

To verify the isolation of primary endothelial cells from the human umbilical vein, cells were tested for the expression of the endothelial marker platelet/endothelial cell adhesion molecule-1 (PECAM-1). PECAM-1 was expressed at cell-cell-contacts and thus identified HUVEC (Fig. 3.3).

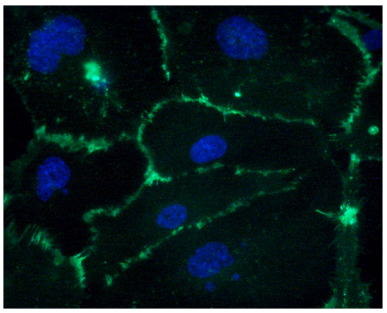


Fig. 3.3: Primary cells of human umbilical cord isolates are positive for PECAM-1. Immunofluorescence staining of primary isolated cells from the human umbilical vein for the endothelial marker PECAM-1 (green) to verify endothelial origin. Cells were positive for PECAM-1, which was localized at cell-cell-contacts. DNA was stained with Hoechst (blue).

3.1.2 Reversible immortalization: Testing the system with Hela and TMNK-1 control cells

In order to generate reversibly immortalized primary human cells as a new infection model, the effects of three different oncogenes were analyzed in this study. Depending on the activity of the respective oncoprotein, cellular immortalization will lead to different phenotypic changes within the cells. Oncogenic mechanisms include senescence bypass by elongation of telomeres (by hTERT) as well as inhibition of tumor suppressors. The latter can take place through protein-interactions (by SV40T) or on transcriptional level (by Bmi1). The oncoproteins' characteristics are summarized in table 3.1. They have been used for the intended immortalization of various epithelial cells before (see introduction, chapter 1.5.1 ff.). In the following study expression of single oncogenes as well as combination of two oncogenes were tested to immortalize primary human cells.

Tab. 3.1: Characteristics of the oncoproteins used in this work. For details and references, see chapter 1.5.1 ff. in the introduction.

Oncoprotein	Full name	Origin	Function
hTERT	Human telomerase reverse transcriptase	human	- catalytic subunit of human telomerase - elongation of telomeric repeats at end of chromosomes to counteract senescence
SV40T	Simian Virus 40 large T antigen	viral	- inactivation of retinoblastoma proteins, which leads to activation of transcription factors and entry into S phase of cell cycle - inactivation of p53 tumor suppressor - protein necessary for the viral DNA replication and transcription
Bmi1	B lymphoma Mo-MLV insertion region 1	mouse (human homolog exists)	- transcriptional repressor - repression of the ink4a-locus with the tumor suppressors p16 and p19Arf (human homolog: p14Arf) - a polycomb ring finger protein, required for self-renewal of adult stem cells

For oncogene transfer into primary cells, a lentiviral based system was implemented with gene delivery through virus like particles – a process also called viral transduction. The lentiviral system used in this work is derived from Human Immunodeficiency Virus 1 (HIV-1). Lentiviruses are able to infect dividing as well as non-dividing cells and therefore are highly suitable for infection of primary cells, which do not always grow in culture. Delivered genes are integrated into the host genome, which enables long-term and stable expression.

Fig. 3.4 gives an overview over the strategy for reversible immortalization via lentiviral gene transfer. Recombinant lentivector particles (LV particles), which encode one of the immortalizing oncoproteins hTERT, SV40T or Bmi1, were used for gene transfer into the human target cells.

For production of LV particles (Fig. 3.4A), three plasmids are co-transfected into producer cells, e.g. 293T (human embryonic kidney cells). The plasmids encode the viral packaging system, the oncoprotein and the envelope protein. The envelope protein from Vesicular Stomatitis Virus (VSV) is used due to its high stability and broad tissue tropism. The other structural and enzymatic components of the LV particles are derived from HIV-1. The viral components are expressed in the producer cells and assemble to LV particles carrying the oncogene. The oncogene-construct itself is the only genetic material that will be incorporated into the LV particles. Therefore, no genetic information coding for genes from HIV or VSV are transferred. LV particles are released into the cell supernatant through budding from the cytoplasmic membrane.

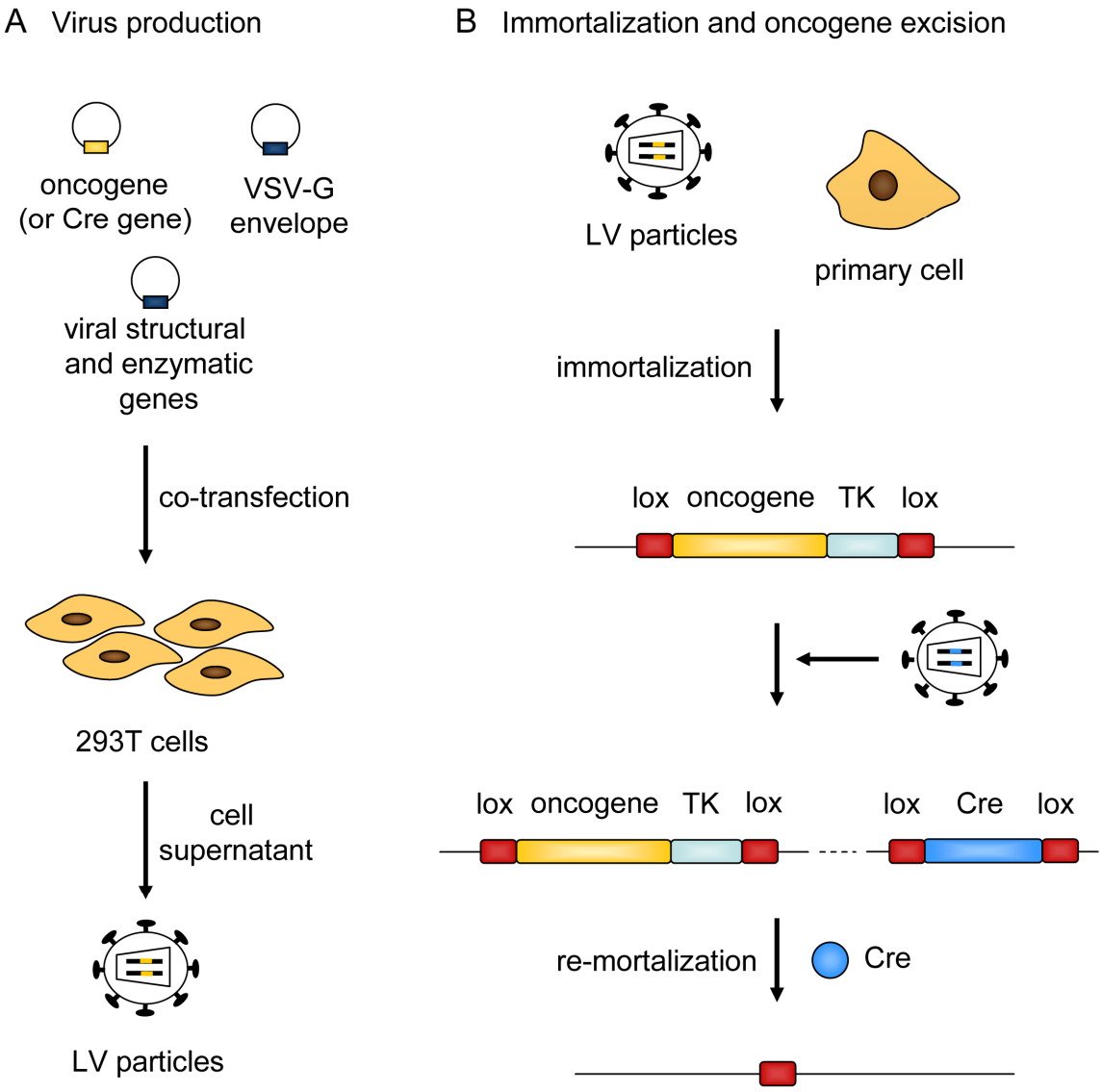
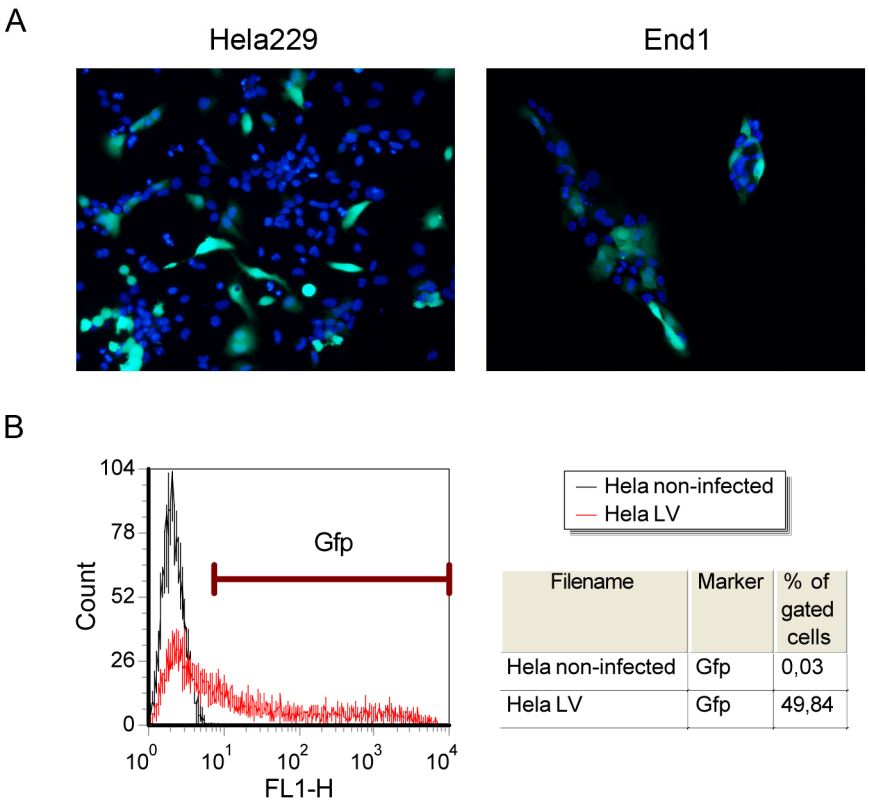


Fig. 3.4: Strategy for the reversible immortalization via lentiviral gene transfer. Scheme of reversible immortalization via lentiviral gene transfer. **A)** For production of recombinant lentivector (LV) particles, three plasmids are co-transfected into 293T producer cells. The plasmids contain the target gene (encoding for oncoprotein hTERT, SV40T or Bmi1 or for recombinase Cre), the gene for the viral envelope protein (Vesicular Stomatitis Virus Glycoprotein, VSV-G) and the viral structural and enzymatic genes. Produced LV particles are released into the cell supernatant. **B)** For immortalization, primary cells are infected with recombinant LV particles carrying the oncogenes. These target genes are flanked by lox-sites after integration into the cell's genome. Re-mortalization (excision of oncogenes) is mediated by the recombinase Cre. Therefore, immortalized cells are superinfected with LV particles encoding Cre. After integration, expressed Cre recombinase recognizes the lox-sites and excises the integrated genes. To select re-mortalized cells, the viral thymidine kinase (TK) suicide gene is present on the immortalizing plasmids in addition to the oncogen for negative selection of immortalized cells. For details, see text and materials and methods.

For immortalization, primary cells are infected with the generated LV particles (Fig. 3.4B). After infection, the oncogenes integrate into the genome of primary cells and are expressed under the control of an active viral promoter. After integration, the oncogenes

are flanked by lox-sites at both ends. Excision of oncogenes, i.e. re-mortalization, is mediated by infection of immortalized cells with LV particles encoding the recombinase Cre. This enzyme recognizes the lox-sites and excises the oncogenes between the two lox-sequences. As the gene encoding the recombinase is flanked by lox-sites, too, this sequence is excised as well.
For selection of re-mortalized cells with excised constructs, the viral thymidine kinase (TK) suicide gene from Herpes Simplex Virus type 1 (HSV-1) is used. Cells which still carry the constructs after Cre treatment die upon addition of the substrate analogon ganciclovir to the medium. HSV-1 TK is encoded additionally on the immortalizing constructs with hTERT and SV40T genes.

The LV particles carrying either one of the oncogenes or the recombinase gene were produced as explained in Fig. 3.4A. To confirm that produced LV particles were infective in cell culture and to get an estimation of infection rates, standard cell lines were infected with the Gfp-encoding LV particles. Hela229 cells (human epithelial cervix carcinoma cells) and End1 cells (human epithelial endocervical cells) were infected with a multiplicity of infection (MOI) 1 (Fig. 3.5A). Infection was verified by immunofluorescence analysis with staining for Gfp. Approximately ¼ of the cells were Gfp-positive. The Gfp-positive infected cells were detected in both cell lines throughout samples. This confirmed both successful production of LV particles and efficient infection.
For quantification of the infection rate, Hela229 cells were infected with the same Gfp-encoding LV particles. Infected cells were analyzed for the Gfp-positive population in comparison to non-infected cells 5 days post infection (p.i.) (Fig. 3.5B). An infection rate of approximately 50% was observed in the Hela229 cells infected at MOI1. These analyses show that Gfp-encoding LV particles infected standard cell lines efficiently. The corresponding oncogene-carrying LV particles therefore were used for the immortalization of primary cells.



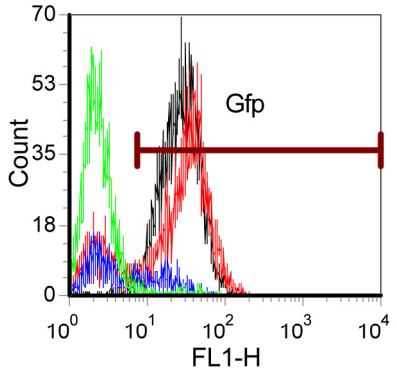
Filename	Marker	% of gated cells
Hela non-infected	Gfp	0,03
Hela LV	Gfp	49,84

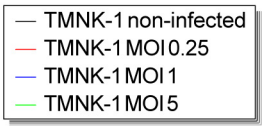
Fig. 3.5: Recombinant Gfp-encoding LV particles infect cell lines efficiently. A) Verification of infection by immunofluorescence analysis. Hela229 and End1 cells were infected with Gfp-encoding LV particles with a multiplicity of infection (MOI) 1 and analyzed 3 days post infection (p.i.). Samples were stained for Gfp (green) and with Hoechst (DNA, blue). Gfp-positive infected cells were detected in both cell lines throughout samples. **B)** Quantification of infection rate by FACS flow cytometry analysis. Hela229 cells infected with Gfp-encoding LV particles were analyzed for the Gfp-positive population in comparison to non-infected cells 5 days p.i.. An infection rate of approx. 50% was observed in Hela229 cells infected at MOI1. In the histogram, cell counts are plotted against fluorescence intensity measured in the FL-1 channel. The red bar designates the Gfp-positive cell population.

The system of reversible immortalization using lentiviral gene transfer was tested in a standardized cell line, in which the method was known to work. This cell line, TMNK-1, is derived from human liver endothelial cells through immortalization with hTERT and SV40T via retroviral gene transfer (Matsumura, et al., 2004). One of the immortalizing constructs used by Matsumura and colleagues additionally encodes Gfp. The immortalizing constructs are both flanked by lox-sites and can be excised by Cre-recombinase. Oncogene excision had been shown using a purified Cre-fusion protein (Matsumura, et al., 2004). We altered the method by using lentiviral delivery of the Cre recombinase gene. Thus, TMNK-1 cells were optimal control cells to study re-mortalization using the lentiviral system.

To test the efficiency of oncogene excision (re-mortalization) in the lentivector system, TMNK-1 cells were infected with LV particles encoding the Cre-recombinase (Fig. 3.6A).

A





Filename	Marker	% of gated cells
TMNK-1 non-infected	Gfp	98,26
TMNK-1 MOI 0.25	Gfp	77,99
TMNK-1 MOI 1	Gfp	34,3
TMNK-1 MOI 5	Gfp	2,34

B

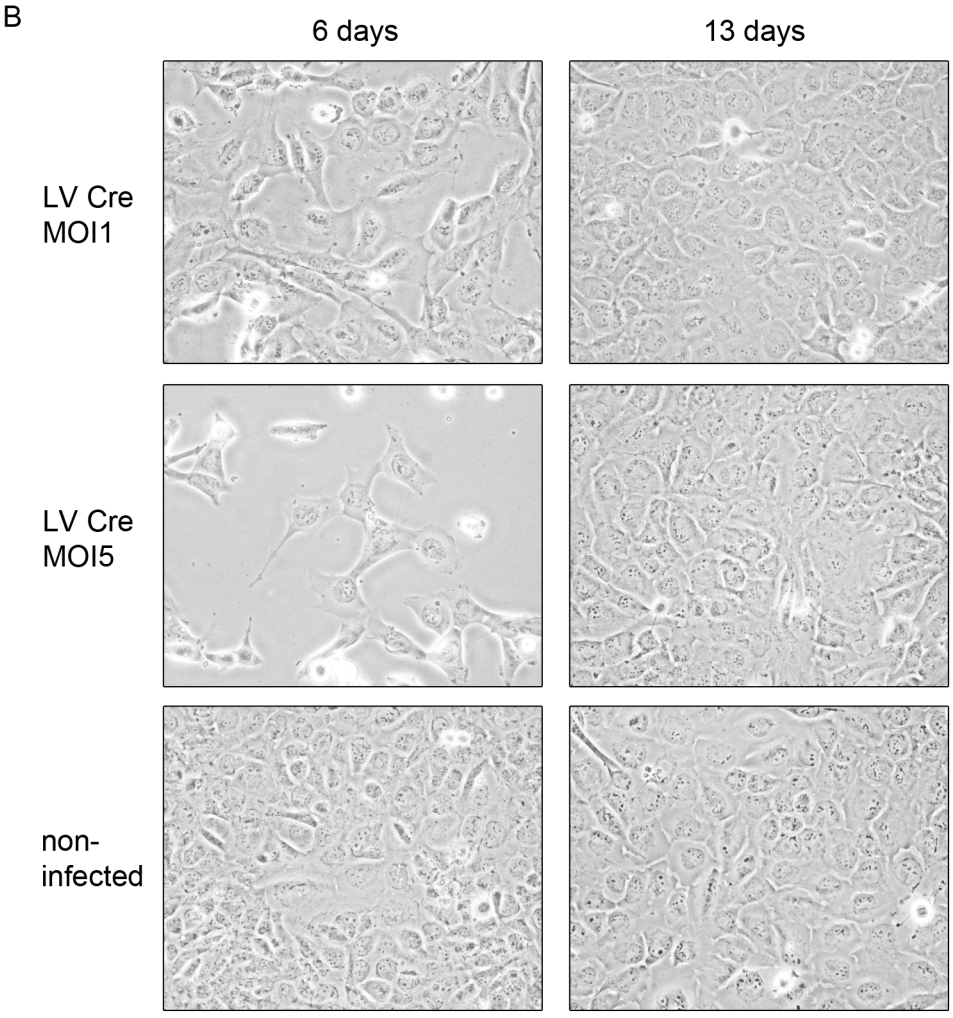


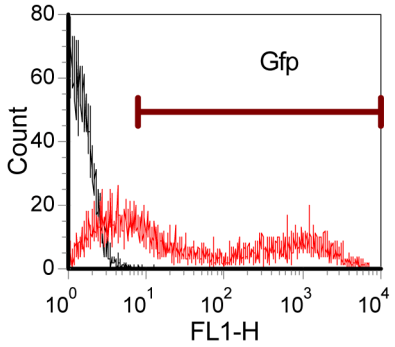
Fig. 3.6: Quantification of oncogene excision (re-mortalization) and analysis of cell phenotype after oncogene excision in control cell line TMNK-1. A) Oncogene excision (re-mortalization) via Cre-encoding LV particles is efficient in the control cell line TMNK-1. The cells were infected with LV particles encoding the Cre-recombinase using different MOI to test efficiency of oncogene excision. Samples were analyzed in flow cytometry 6 days p.i. for the Gfp-positive population, which decreased with increasing MOI. The bar designates the Gfp-positive cell population. Cell counts were plotted against fluorescence intensity. **B)** MOI-dependant survival of TMNK-1 control cells infected with Cre-encoding LV particles (LV Cre). TMNK-1 cells continuously grow after Cre-mediated excision of oncogenes. Cells infected with MOI 1 showed higher survival rate than cells infected with MOI 5. The cells were infected with LV Cre and analyzed 6 and 13 days p.i..

Successful gene excision by Cre-recombinase will remove oncogene constructs including *GFP* from TMNK-1 cells, and therefore will reduce the fraction of Gfp-positive cells. Samples were analyzed six days p.i.. The Gfp-positive population decreased with increasing MOI of Cre-encoding LV particles. Using MOI 0.25, approximately 78 % Gfp-positive cells remained. MOI 1 and MOI 5 led to approx. 34 % and 2 % Gfp-positive cells left, respectively. These results demonstrated efficient gene excision by Cre-recombinase. For evaluation of viability and cell phenotype after oncogene excision, TMNK-1 cells were infected with Cre-encoding LV particles (Fig. 3.6B). Cells were analyzed 6 and 13 days p.i.. After re-mortalization, both MOI 1 and MOI 5 infected samples showed diminished cell numbers compared to non-infected cells 6 days p.i.. However, only few cells died after infection with MOI 1 compared to non-infected cells. Surviving cells recovered fast. At MOI1, no significant increase in cell death or negative impact on cell growth was detected. In contrast, levels of cell death were increased after infection with MOI 5 and remaining cells grew slower. These samples recovered confluence only with time delay. Therefore, TMNK-1 cells in general grow continuously after Cre-mediated excision of oncogenes. According to cell recovery, MOI 1 as well as MOI 5 can be used, but MOI5 has a negative effect on cell survival. Infection with Cre-encoding lentiviruses with MOI 1 led to 2/3 of the cells having excised constructs while a high survival rate was maintained.

3.1.3 Immortalization of primary endothelial cells (HUVEC) using viral gene transfer and characterization of immortalized cells

Recombinant Gfp-encoding LV particles could be shown to infect standard cell lines efficiently (see Fig. 3.5). These LV particles were produced using the same plasmid background as the oncogene-carrying LV particles needed for immortalization. The next step was to test the infection efficiency in primary cells.

Primary HUVEC (human umbilical vein endothelial cells) were infected with Gfp-encoding LV particles and the infection rate was quantified. For this purpose, samples were analyzed for the Gfp-positive population 5 days p.i. (Fig. 3.7). The primary HUVEC were infected with MOI 1.6. An infection rate of approximately 61 % was observed. This experiment showed efficient infection of primary cells by the recombinant Gfp-encoding LV particles. The result is comparable to the infection rate in Hela229 cells (see Fig. 3.5) with 50 % infected cells using MOI 1.



Filename	Marker	% of gated cells
HUVEC non-infected	Gfp	0,04
HUVEC LV	Gfp	61,19

Fig. 3.7: Recombinant Gfp-encoding LV particles infect primary HUVEC efficiently. Quantification of infection rate by FACS analysis. Primary HUVEC infected with Gfp-encoding LV particles were analyzed for the Gfp-positive population in comparison to non-infected cells 5 days p.i.. An infection rate of approx. 61 % was observed in primary HUVEC infected with MOI 1.6. The histogram displays cell count against fluorescence intensity. The red bar designates the Gfp-positive cell population.

The next aim was to generate immortalized HUVEC by using the oncoproteins hTERT, SV40T and Bmi1 to analyze the effects of these oncoproteins on primary cells. All three oncoproteins were used individually as well as in combination of two each. Primary HUVEC were infected in parallel with LV particles carrying the different oncogenes with approximately MOI 1 and grown continuously. Four months after infection with LV particles, pictures were taken to visualise different growth characteristics. Cells expressing hTERT + SV40T were rounder and more regular shaped compared to all other samples (Fig. 3.8). Among the cells transduced with one of the oncogenes individually, only SV40T expressing cells continued proliferating beyond passage 29. Thus, hTERT or Bmi1 alone were not sufficient for immortalization. However, comparing SV40T expressing cells with double-transduced cells could indicate incomplete immortalization, as SV40T expressing cells grew less steadily and more cells were enlarged or died. Four samples of transduced cells survived: HUVEC immortalized with hTERT + SV40T, hTERT + Bmi1, SV40T + Bmi1 and SV40T alone. The cells were cultivated up to p. 50 before original continuous culture was stopped.

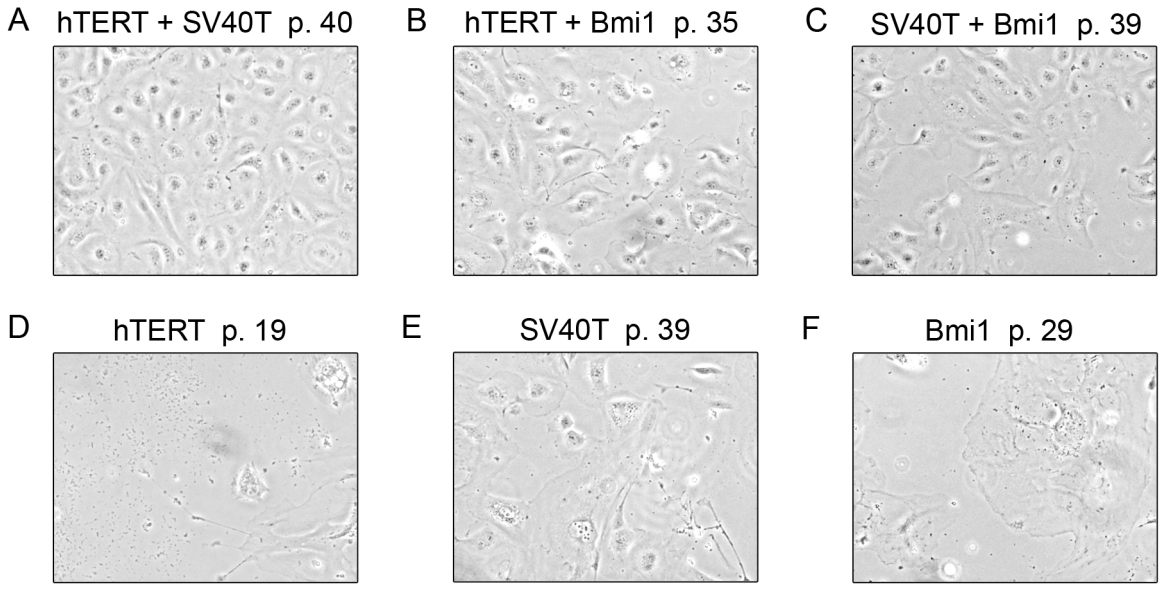


Fig. 3.8: Differences in cell viability and phenotype of immortalized HUVEC due to combination of oncogenes. Phase contrast pictures of transduced HUVEC four months post transduction. Primary HUVEC cells were infected with LV particles carrying different oncogenes as indicated and grown in parallel continuously. Pictures were acquired on the same day and passage numbers (p.) are indicated to visualise different growth characteristics. **A)** HUVEC immortalized with hTERT + SV40T grew steadily and had a round phenotype. **B)** HUVEC with hTERT + Bmi1 grew continuously, they exhibited longer and more irregular cell shapes. **C)** HUVEC with SV40T + Bmi1 grew steadily. Many cells appeared round with long cell shapes in between. **D)** HUVEC with hTERT alone had lowest survival rates. Cells had stopped growing in p. 19 and died completely after additional step of subculture. **E)** HUVEC with SV40T alone grew continuously. Cells showed mixed phenotypes with long as well as irregular cell shapes. **F)** HUVEC with Bmi1 alone had diminished survival rates. Enlarged irregular cells were observed, which died after additional step of subculture.

The successfully growing transduced HUVEC were considered to be immortalized. Next, gene expression of the oncogenes was verified in HUVEC immortalized with two of the oncogenes. The oncogene expression according to the inserted constructs could be verified in all three HUVEC samples transduced with two oncogenes (Fig. 3.9). Primary HUVEC did not express *HTERT*, *SV40T* or *BMI1*. *HTERT* was expressed in all HUVEC transduced with the hTERT construct (Fig. 3.9A). HUVEC immortalized with hTERT + SV40T showed 1.6-fold higher *HTERT* expression level than HUVEC immortalized with hTERT + Bmi1. All HUVEC carrying the SV40T construct showed expression of *SV40T* (Fig. 3.9B). HUVEC immortalized with hTERT + SV40T showed a 3.6-fold lower expression level than HUVEC immortalized with SV40T plus Bmi1. Both HUVEC immortalized with Bmi1 construct expressed *BMI1* (Fig. 3.9C). However, *BMI1* expression was 16.5-fold lower in HUVEC immortalized with hTERT + Bmi1 compared to HUVEC immortalized with SV40T + Bmi1.

Notably, HUVEC immortalized with SV40T + Bmi1 showed highest expression of both transduced oncogenes *SV40T* and *BMI1* among all HUVEC. TMNK-1 cells served as

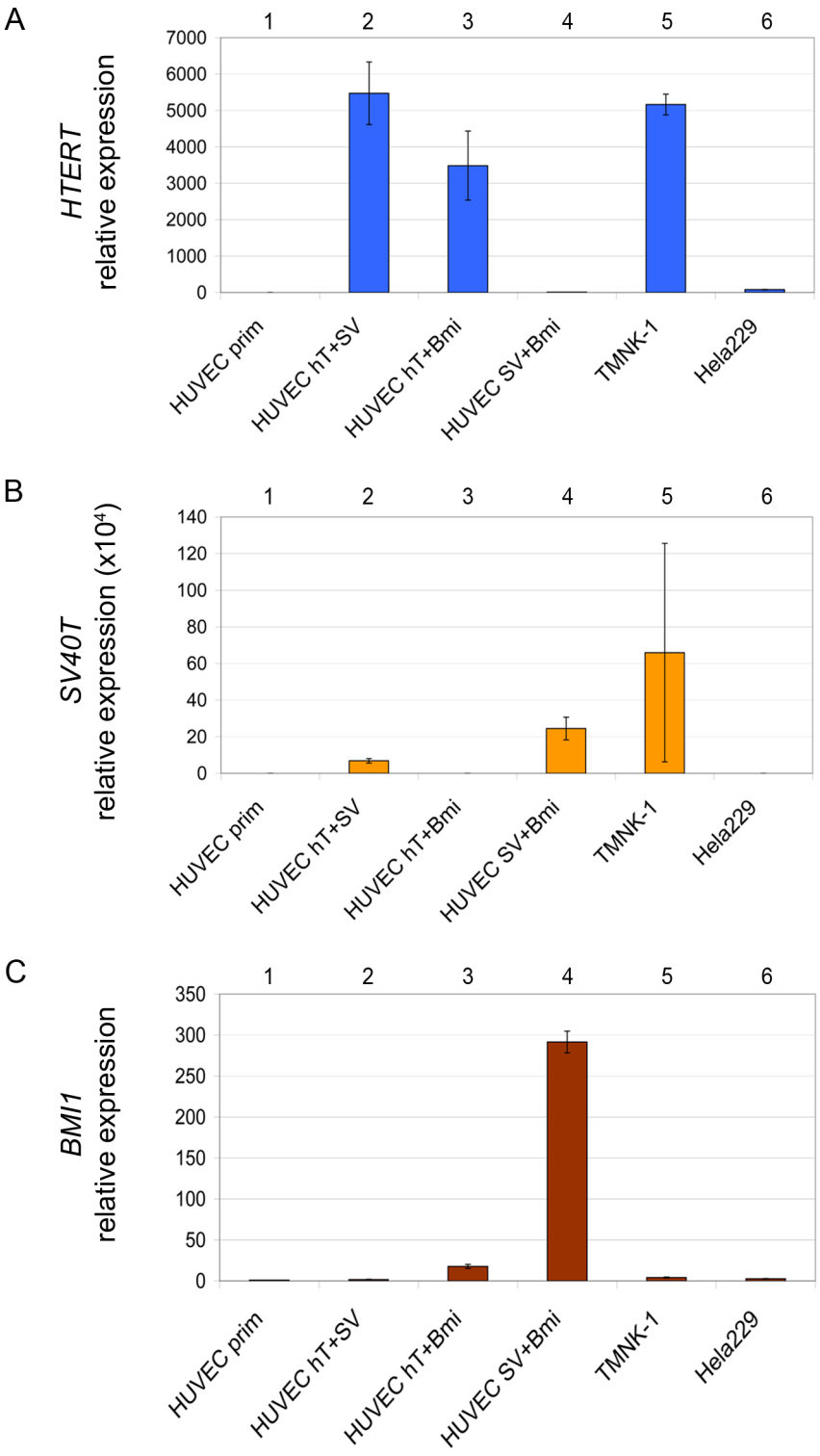


Fig. 3.9: Verification of oncogene expression in immortalized HUVEC by real time RT-PCR. Quantitative real time RT-PCR was performed to determine the expression of oncogenes on mRNA level in immortalized HUVEC cells compared to primary HUVEC cells, TMNK-1 cells and Hela229 cells. Relative expression values were normalized to primary HUVEC (sample 1). hT: hTERT; SV: SV40T; Bmi: Bmi1. **A)** Relative expression of *HTERT*. *HTERT* was expressed in all HUVEC transduced with the hTERT construct (samples 2, 3) and in TMNK-1 cells. **B)** Relative expression of *SV40T*. All HUVEC carrying the SV40T construct (samples 2, 4) and TMNK-1 cells showed expression of *SV40T*. **C)** Relative expression of *BMI1*. HUVEC carrying Bmi1 construct expressed *BMI1* (samples 3, 4). In other cells, only basal *BMI1* expression was detected.

positive control and expressed *HTERT* as well as *SV40T* as expected (see above). Hela229 cancer cells expressed *HTERT* at very low levels (64-fold lower compared to TMNK-1 *HTERT* expression). As oncogene expression was verified for the respective genes, the immortalization of primary HUVEC was successful.

After immortalization of primary cells, characterization of obtained cells was necessary to verify endothelial origin and to exclude changes of endothelial phenotype. Therefore, the four surviving immortalized HUVEC samples were analyzed for expression of the endothelial marker platelet/endothelial cell adhesion molecule (PECAM-1) (Fig. 3.10). All cell samples were positive for PECAM-1, which localized at cell-cell-contacts, and thus all cells could be confirmed as endothelial cells. The localization of PECAM-1 corresponds to the one in primary HUVEC (compare Fig. 3.3).

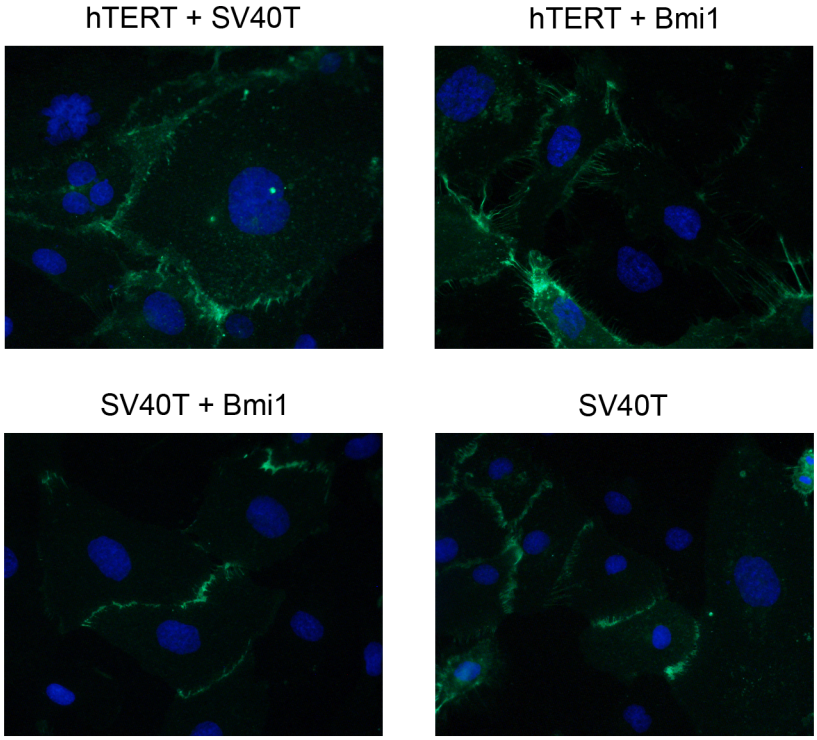


Fig. 3.10: Immortalized HUVEC express endothelial marker PECAM-1. Immunofluorescence staining of immortalized HUVEC cells for the endothelial marker PECAM-1 (green) to verify endothelial origin. DNA was stained with Hoechst (blue). Oncoproteins used for immortalization are indicated. Immortalized cells were positive for PECAM-1, which localized at cell-cell-contacts between confluent cells. Absence of PECAM-1 signal in some cells can be explained by lack of confluence.

3.1.4 Excision of oncogenes using Cre recombinase

Cells carrying constructs for reversible immortalization can be reverted back to a “primary-like” phenotype by cutting the oncogenes out of the genome. Such re-mortalized cells need to be healthy and ideally growing actively, to be suitable for subsequent experiments. In the system applied in this study, selection of re-mortalized cells with excised oncogene constructs is obtained by negative selection of cells which still carry the oncogenes. This is achieved through Herpes Simplex Virus type 1 thymidine kinase (HSV-1 TK) (compare Fig. 3.4), which is encoded on hTERT and SV40T constructs. *HSV-1 TK* is a suicide gene, because the kinase renders the cells sensitive to ganciclovir, which eventually leads to cell death (Salmon, et al., 2000; Caruso, 1996). To exclude general toxicity, primary HUVEC used in this study were treated with ganciclovir. The cells were not affected, as their growth characteristics did not change during the observed time period.

In order to test oncogene excision, verify the excision and check cell condition afterwards, immortalized HUVEC were infected with Cre-encoding LV particles (LV Cre) to excise the oncogenes. The same MOI was used that had been efficient in TMNK-1 cells while a high survival rate had been maintained. As controls, samples not infected with LV Cre were kept in parallel. All cells were treated with ganciclovir to kill cells with non-excised constructs. Cells not infected with LV Cre were expected to die upon addition of ganciclovir. In LV Cre infected samples, all cells with successful and efficient oncogene excision would survive. At 13 days p.i., all samples infected with Cre-encoding LV particles and treated with ganciclovir exhibited enlarged cells and were reduced in cell numbers (Fig. 3.11A). Growth arrest occurred in all samples. Non-infected cells treated with ganciclovir died completely in three of four samples. This indicates that oncogene constructs were excised in the respective LV Cre infected cells. Only non-infected HUVEC carrying hTERT + Bmi1 were less sensitive to ganciclovir. As control, Fig. 3.11B shows untreated HUVEC without ganciclovir immortalized with hTERT + SV40T for comparison. These cells continued normal growth in contrast to the ganciclovir treated re-mortalized HUVEC, which arrested growth.

To elucidate possible reasons for the growth arrest of HUVEC after Cre treatment, the samples were stained at the end of the Cre experiment for dead cells. In this preliminary staining experiment, the Cre-treated HUVEC samples did not have elevated levels of cell death compared to untreated samples. This finding indicated that growth arrested cells did not undergo cell death above basal level after excision of oncogenes.

The conclusion of this experiment is that re-mortalization of immortalized HUVEC is principally possible, as oncogene excision was achieved. However, re-mortalized cells arrested growth, which might indicate diminished cell fitness. Follow-up experiments

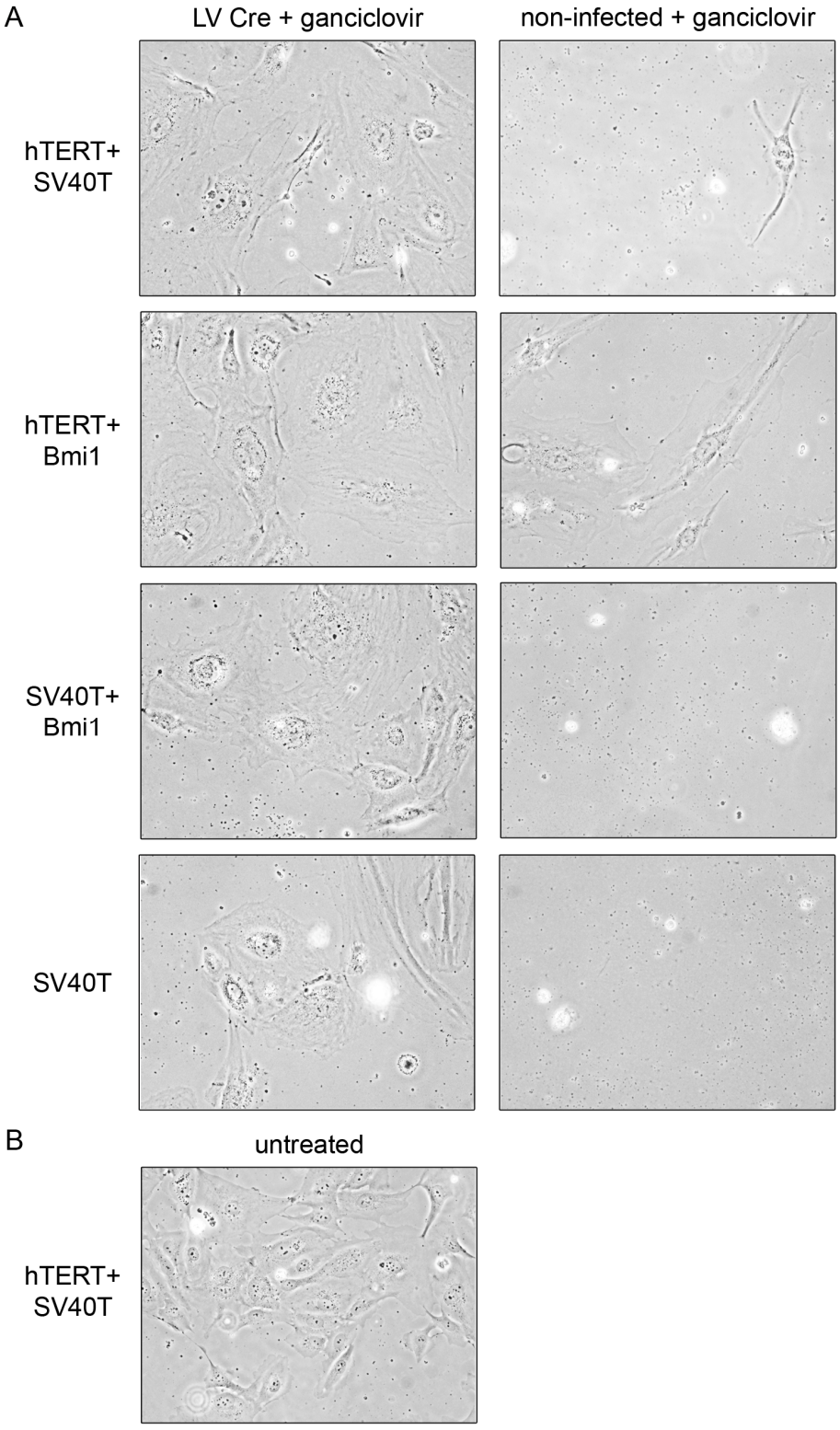


Fig. 3.11: Verification of oncogene excision by ganciclovir treatment in immortalized HUVEC. A) Immortalized HUVEC cells (respective oncoproteins indicated) were infected with Cre-encoding LV particles (LV Cre) at MOI 1 to excise the oncogenes. Cells were analyzed 13 days p.i.. Three days after infection with LV Cre, the cells had been treated with 1 µM ganciclovir for 10 days to kill cells with non-excised constructs. All samples infected with Cre-encoding LV particles and treated with ganciclovir (left) showed enlarged cells and reduced cell numbers. Growth arrest occurred. Oncogene constructs were excised in these cells, as non-infected cells treated with ganciclovir (right) died. **B)** Untreated HUVEC immortalized with hTERT + SV40T for comparison.

studying host-pathogen interactions can take several days. Therefore it is questionable whether the system of reversible immortalization can be used, as re-mortalized HUVEC arrested cell growth already during the reversion process.

3.1.5 Immortalization of primary fallopian tube cells using viral gene transfer

The recombinant oncogene-carrying LV particles had been shown to successfully immortalize primary HUVEC. These LV particles were now used to generate immortalized cell lines from fallopian tubes, which are a natural site of infection for genital tract pathogens, to study host-pathogen-interactions.

Single oncoproteins (hTERT, SV40T or Bmi1) were used initially to immortilize primary FT cells, but none of the samples grew continuously and no stably transduced cell lines with only one oncogene could be obtained. Therefore, combinations of two of the oncoproteins hTERT, SV40T and Bmi1 were used as in the previous experiments, where these combinations had been successful in the immortalization of primary HUVEC.

Primary fallopian tube cells showed signs of cellular stress after LV particle infection. Fig. 3.12 shows primary FT cells 3 days post LV particle infection, i.e. oncogene transduction. The transduced cells showed stress fibres and vacuoles. More stressed cells with stress fibres and accumulated vacuoles were observed in samples transduced with hTERT- + Bmi1-encoding LV compared to combinations with SV40T.

Initially, epithelial cells should be selected of mixed primary populations before lentiviral infection; however, this approach was not followed up, as the cells mostly died after subculture. Due to the reduced growth potential of primary cultures, infection was performed directly in primary isolates in average 1 week after isolation. Cell numbers of starting samples were usually rather low, in the range of only few hundred cells per

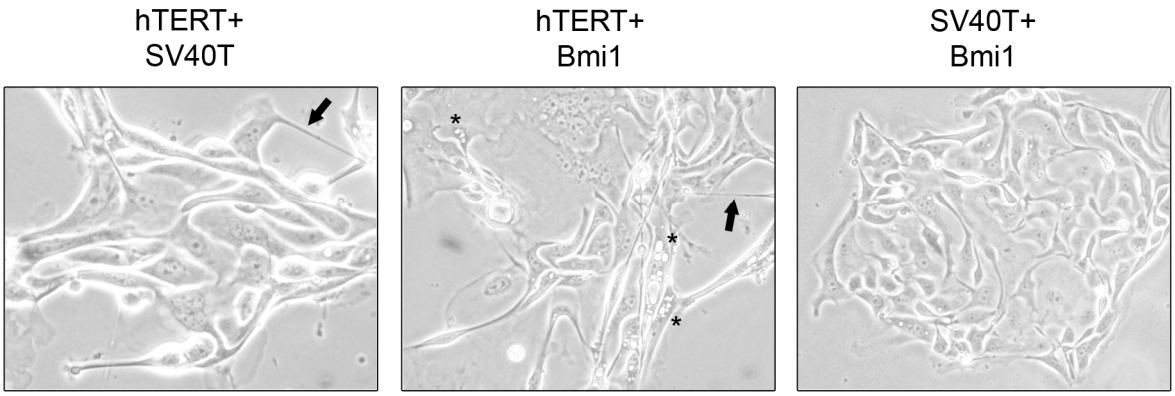


Fig. 3.12: Cellular stress phenotypes in primary fallopian tube culture after lentiviral infection. Phase contrast pictures of primary fallopian tube cells 3 days post viral infection (oncogene transduction). Combinations of used constructs are indicated. Cells partially showed stress fibres (examples marked by arrow) and vacuoles (examples marked by asterisk). Cells transduced with hTERT- plus Bmi1-constructs developed stress fibres and vacuoles more often.

sixwell. Cells had not reached complete confluence when used for oncogene transduction. In total, primary isolates of 18 tissue pieces (derived from 12 different patients) were used in 10 independent immortalization experiments.

Immortalized samples consisting of mixed populations were cloned by using cloning rings or FACS single cell sorting. All obtained FT cell clones, which were continuously growing, were immortalized with SV40T and Bmi1. Cells within transduced samples were growing and expanding only if SV40T was present.

Gene expression of the transduced oncogene constructs was analyzed in the best characterized ones of the obtained clonal FT cell lines (named FT-01 to FT-06; compare chapter 3.1.6) to control immortalization. mRNA levels were compared to three different samples of primary FT cells, which were derived from three different donors (Fig. 3.13). Primary FT cells did not express any of the three oncogenes *HTERT*, *SV40T* or *BMI1*. The immortalized FT cell lines did not express *HTERT*, too, as expected (Fig. 3.13A). All FT cell lines expressed *SV40T*, but expression levels differed (Fig. 3.13B). FT-01, FT-04 and FT-06 cell lines showed strong *SV40T* expression. The FT cell lines FT-02, FT-03 and FT-05 expressed *SV40T* at approximately 4.6-fold lower levels when average values of high-and low-expressing samples were compared.

Unexpectedly, *BMI1* expression levels were lower in FT cells compared to HUVEC (see above) although the same LV particles were used for immortalization. FT-01, FT-04 and FT-06 expressed *BMI1* (Fig. 3.13C). FT-02, FT-03 and FT-05 cells only showed the same basal activity as primary FT cells. Notably, only FT cell lines with high *SV40T* expression (namely FT-01, FT-04 and FT-06) showed *BMI1* expression. TMNK-1 control cells expressed *HTERT* and *SV40T* as expected (see chapter 3.1.2). Hela229 cancer cells expressed *HTERT* at a low level compared to TMNK-1. Both TMNK-1 and Hela cells had low *BMI1* expression when normalized to primary FT cells. The analysis revealed that levels of oncogene expression differed between the obtained FT cells. Expression of both *SV40T* and *BMI1* was verified in cell lines FT-01, FT-04 and FT-06 as expected according to the transduced constructs.

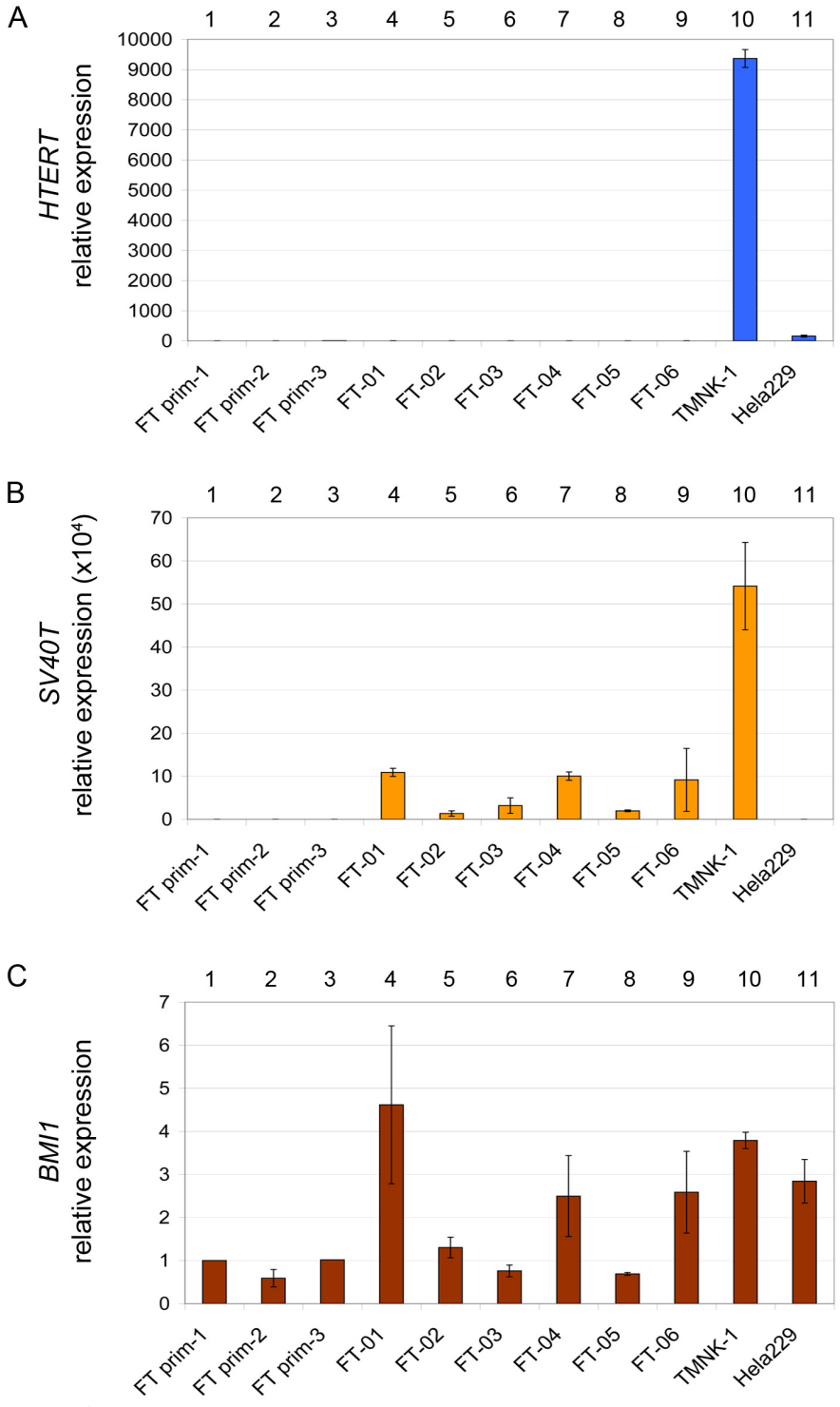


Fig. 3.13: Verification of oncogene expression in immortalized FT cells by real time RT-PCR. Quantitative real time RT-PCR was performed to determine the expression of oncogenes on mRNA level in FT cells immortalized with SV40T and Bmi1 compared to primary FT cells, TMNK-1 and Hela229 cells. Graphs were normalized to primary FT cells (sample 1). **A)** Relative expression of *HTERT*. Neither primary FT cells (samples 1-3) nor FT cells immortalized with SV40T and Bmi1 (samples 4-9) expressed *HTERT*. **B)** Relative expression of *SV40T*. Primary FT cells (samples 1-3) did not express *SV40T*. All FT cell lines immortalized with SV40T and Bmi1 (samples 4-9) expressed *SV40T*. **C)** Relative expression of *BMI1*. *BMI1* was expressed (at least 2-fold compared to primary cells) in FT cell lines FT-01, FT-04 and FT-06 (samples 4, 7 and 9, respectively).

3.1.6 Characterization of immortalized fallopian tube cells

During this work, 21 continuously growing clonal FT cell lines were generated in total, all immortalized with SV40T and Bmi1. Six of these, namely cell lines FT-01 to FT-06, were maintained and observed for a longer period of time. Oncogene expression was verified in these cells (see Fig. 3.13). Two of the clonal lines (FT-01, FT-02) had been obtained by using cloning rings. They both originated from the same donor tissue. The other four clonal FT cell lines had been selected by flow cytometry single cell sorting. Among these, FT-03, FT-04 and FT-05 cells were derived from the same patient sample. FT-06 originated from a third independent donor tissue. For characterization of immortalized FT cells, these six cell clones were analyzed in more detail in the following studies.

Comparison of continuous cell cultures revealed differences in shape and growth pattern (Fig. 3.14). FT-01, FT-02 and FT-06 cells appeared rounded and grew tightly confluent. FT-02 cells were larger than most of the other cells. FT-02 and FT-06 had the most regular growth pattern. FT-03, FT-04 and FT-05 cells had a longer shape. FT-03 and FT-04 cells grew with gaps between cells. FT-05 cells were able to grow tightly confluent as well.

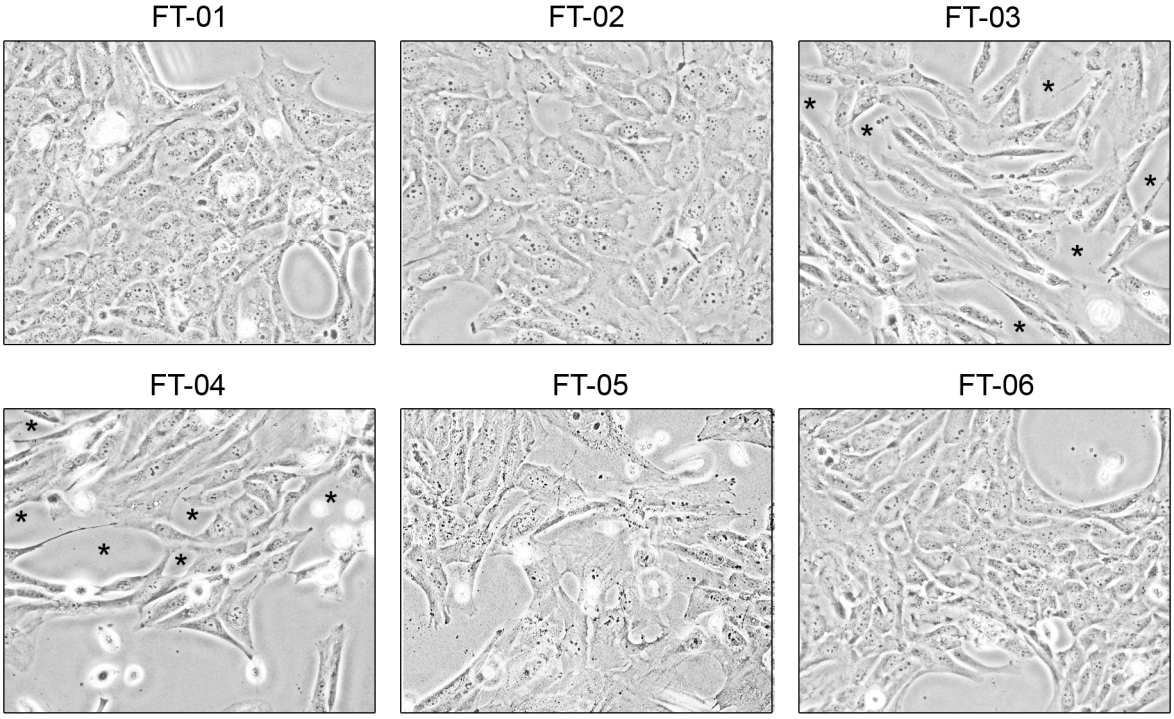


Fig. 3.14: Growth pattern of clonal immortalized FT cell lines. Phase contrast pictures of clonal immortalized FT cell lines immortalized with SV40T and Bmi1. FT-01, FT-02 and FT-06 cells were rounded and grew tightly confluent. FT-03, FT-04 and FT-05 cells showed a longer shape. FT-03 and FT-04 cells grew with gaps (asterisks) between cells. Clones were obtained by cloning rings (FT-01 and FT-02) or by flow cytometry single cell sort (FT-03, FT-04, FT-05 and FT-06). Cells originated from three different patient samples. Patient 1: FT-01 and FT-02; patient 2: FT-03, FT-04 and FT-05; patient 3: FT-06.

The next step was to analyze whether the immortalized cells were able to establish apico-basal cell polarity, as it is observed in polarized epithelial layers. Therefore, FT cell lines were grown on permeable filter supports, which stimulate cells to polarize. Cells were cultivated for approximately two weeks and then analyzed by electron microscopy (EM) (Fig. 3.15). Polarized columnar epithelium, as it is present in FT mucosal folds, is characterized by apico-basal cell polarity, which is maintained by the adhesion belt and functional tight junctions. EM analysis of FT cells revealed multiple cell layers with mainly horizontal orientation of nuclei (Fig. 3.15A). Notably, none of the cell clones showed apico-basal polarity. The number of cell layers and cell forms differed between the cell lines. Flattened disc-like shapes were observed in most samples (FT-01 – FT-04). Of these, FT-01 cells were most flattened and had grown with the highest number of layers on top of each other. Dead cells were observed in the more loosely connected FT-01, FT-03 and FT-04 cells. Rounder cell shapes were found in FT-06 and FT-05 cells. Microvilli were observed at the apical side of cells in the top layer of FT-06 cells, and in lower numbers at FT-02 cells. FT-06 cells appeared most tightly connected of all samples. However, no tight junctions were observed. In conclusion, overall FT cell lines did not show characteristics of polarized epithelial cells.

Notably, among all samples FT-06 cells had the most consistent growth pattern and orientation of cell layers. Closely neighboured cells, which more adhered to each other, were observed in this cell line with nuclei partially in apico-basal orientation (Fig. 3.15B). In addition, these cells were among the FT clones with highest oncogene expression (see above). In T84 control cells, apico-basal polarity was observed, as characterized by vertical nuclei, microvilli and tight junctions (Fig. 3.15C).

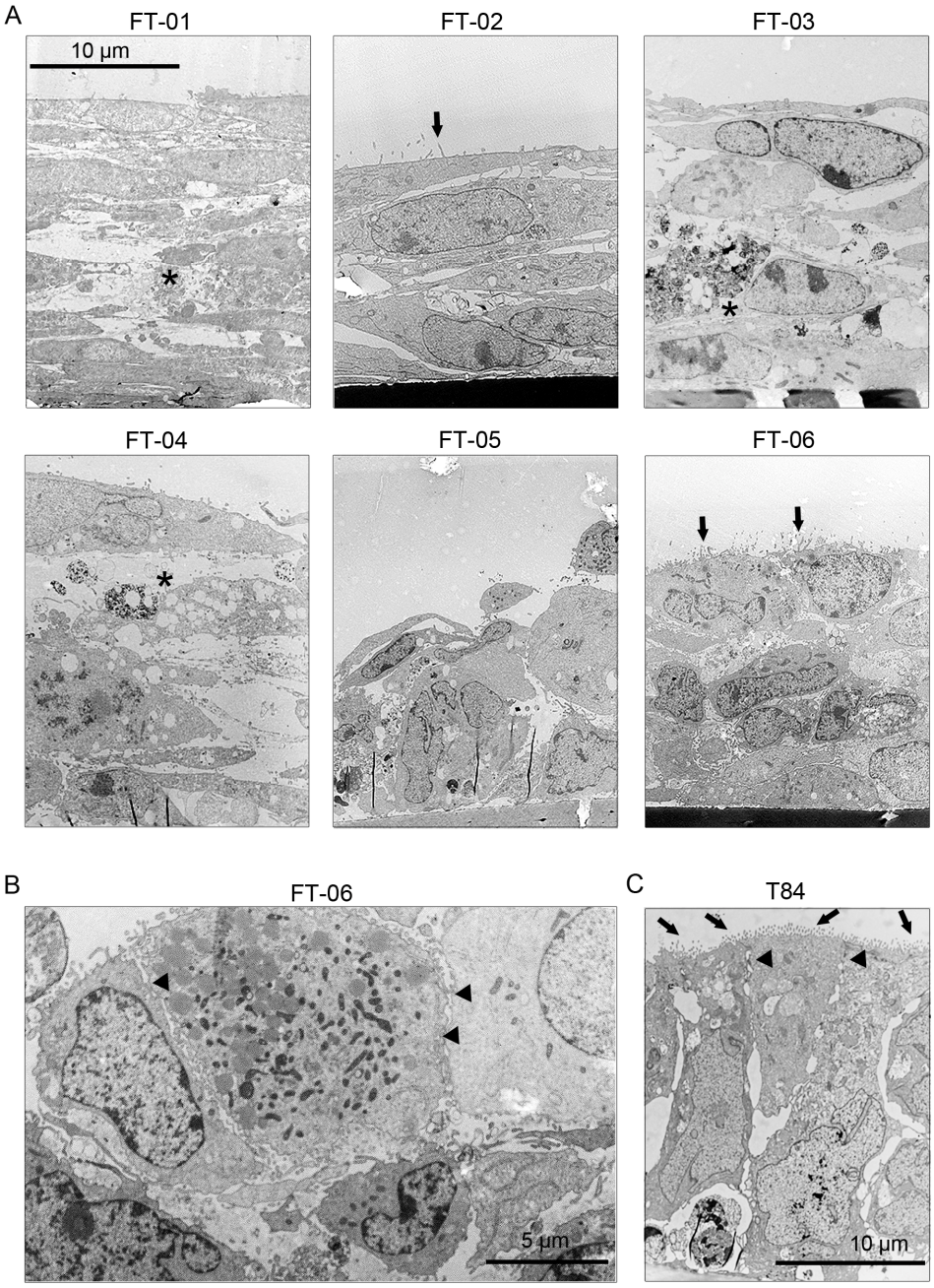


Fig. 3.15: Ultrastructural analysis of FT cell lines reveals differences in cell shape. Cells were grown on permeable filter supports for approximately two weeks to induce cell polarization and then processed for electron microscopy. **A)** FT cells immortalized with SV40T and Bmi1. Notably, none of the cell clones showed apico-basal polarity. Nuclei of cells were oriented horizontally in many cells. No tight junctions or clear adherens juntions were observed. In all samples, multi-layering of cells was observed. The cell lines differed among each other concerning form and number of layers. Most cells had flattened disc-like shapes (FT-01 to FT-04). Of these, FT-01 cells were most flattened and had grown with the highest number of layers on top of each other. Dead cells were observed in the more loosely connected FT-01, FT-03 and FT-04 cells (asterisks). FT-06 and FT-05 cells were rounder. FT-06 cells appeared most tightly connected of all samples. Microvilli were detected at the apical side of cells in the top layer of FT-06 cells (arrows), and to less extent in FT-02 cells (arrow). FT cell lines did not exhibit characteristics of polarized epithelial cells. **B)** Closely neighboured FT-06 cells with close cell contacts (arrowheads) and some nuclei in apico-basal orientation were observed. C) Positive control: T84 cells showed apico-basal polarization expected for epithelial cells with vertically oriented nuclei, microvilli (arrows) and tight junctions (arrowheads). Picture of T84 cells kindly provided by Claudia Lange.

The immortalization of the FT cells was achieved via oncoproteins, some of which are known to be often associated with chromosomal changes, especially SV40T (Ray, et al., 1992). In order to analyze, whether immortalization caused large genomic rearrangements, the chromosomal integrity was tested in the FT cell lines. Therefore, karyotype analysis was performed for the generated cell lines.

All analyzed FT cell lines had abnormal karyotypes with chromosomal changes of both number and structure compared to the normal human diploid chromosome set of 46 chromosomes (Fig. 3.16). FT-06 and FT-03 cells both showed a hypodiploid karyotype with less than 46 chromosomes (Fig. 3.16A). The karyograms revealed missing chromosomes as well as marker chromosomes. Marker chromosomes are structurally abnormal chromosomes, whose origin cannot be identified. The karyotype of FT-06 cells was closest to the normal human female karyotype of 22 chromosome pairs plus 2 X-chromosomes among all cells analyzed. In FT-03 cells ring chromosomes and dicentric chromosomes were found among the marker chromosomes. Dicentric chromosomes are abnormal chromosomes with two centromeres. FT-02 and FT-01 cells both had a polyploid karyotype with triple as well as quadruple chromosomes resulting in high chromosome numbers (Fig. 3.16B). In addition, multiple marker chromosomes were observed. Of these two samples, only FT-02 cells showed single chromosomes, for which the corresponding second copy could not be identified. In some cases, identifiable chromosomes carried changes in form of elongations, e.g. the first chromosome 9 in FT-02 cells and the third chromosome 12 in FT-01 cells (Fig. 3.16B) as well as the second chromosome 12 in FT-06 cells (Fig 3.16A).

The results confirmed chromosomal changes in the immortalized FT cell lines, presumably induced through SV40T. Unfortunately, within the scope of this study immortalization of FT cells was achieved only in the presence of SV40T, as other oncogenes proved to be insufficient for immortalization (see above).

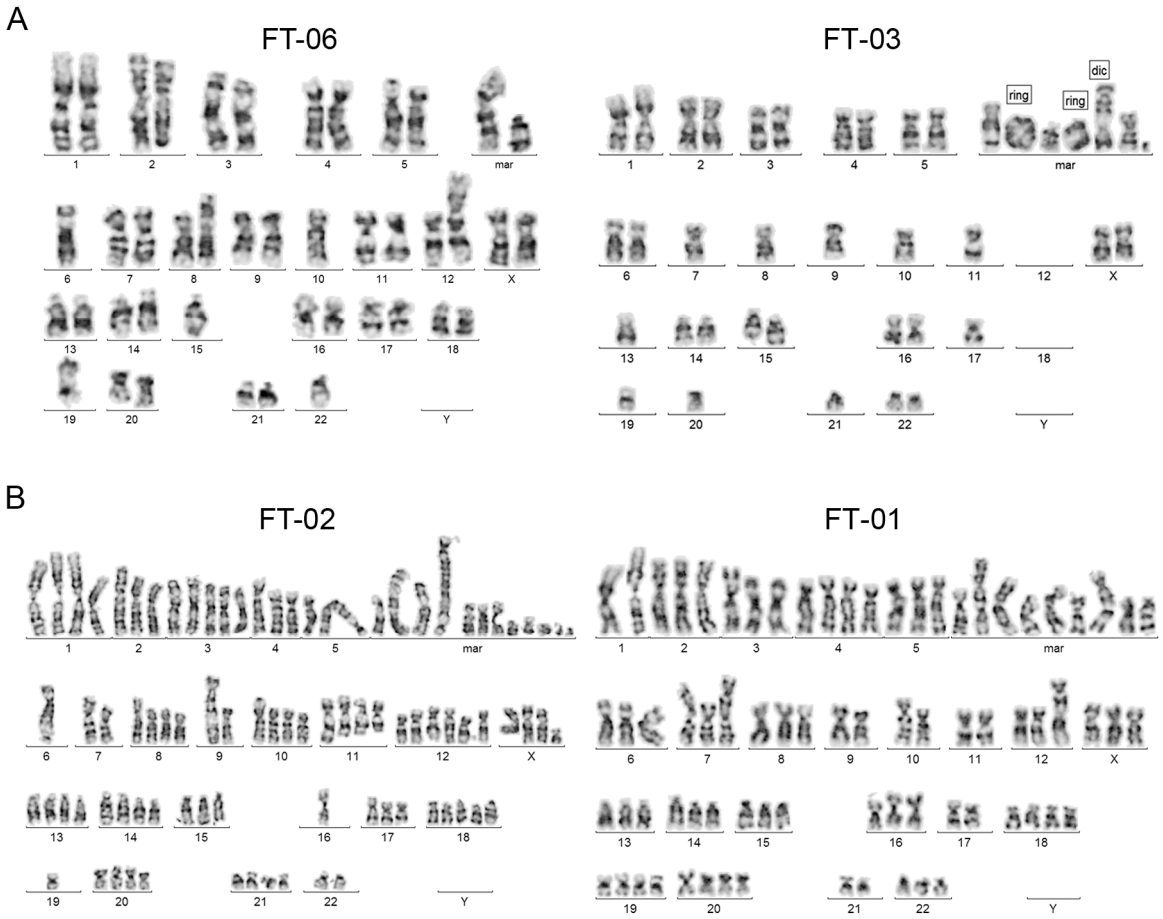


Fig. 3.16: Extensive chromosomal changes are revealed in FT cell lines by karyotype analysis. Karyograms of FT cell lines immortalized with SV40T and Bmi1. All analyzed FT cell lines had abnormal karyotypes. **A)** FT-06 and FT-03 cells showed a hypodiploid karyotype with missing chromosomes as well as marker chromosomes (mar; top right in each karyogram). The karyotype of FT-06 cells (left) was closest to the normal human female karyotype. FT-03 cells (right) showed ring chromosomes (ring) and dicentric chromosomes (dic), which are abnormal chromosomes with two centromeres, among marker chromosomes. **B)** FT-02 and FT-01 cells had a polyploid karyotype with multiple marker chromosomes as well as triple and quadruple chromosomes. Only FT-02 cells (left) showed single chromosomes.

Since immortalization was performed in original isolates consisting of mixed cultures, the origin of generated cell lines needed to be confirmed by the analysis of expression of cell type specific markers. In the following, FT-06 cells are shown as a typical example. These cells appeared least changed of all analyzed FT cell lines in their morphology and karyotype and were among the cells with highest oncogene expression.

FT cell lines were compared to the epithelial cell line End1 to investigate whether FT cells were of epithelial origin (Fig. 3.17). Confluent cells were stained for three epithelial markers: epithelial cell adhesion molecule (EpCAM), E-cadherin and cytokeratin-8. FT-06 cells, which are shown as example, did not express the adhesion molecule EpCAM, whereas in End1 cells EpCAM was widely expressed and localized mainly to the cell borders (Fig. 3.17, top). Cadherins are a component of adherens juntions and E-cadherin

is the subtype found in epithelial cells. However, E-cadherin was only found at cell-cell-contacts of End1 cells, but not in FT-06 cells (Fig. 3.17, middle). Cytokeratin-8 is a cytoskeleton component in epithelial cells derived from simple columnar epithelium, as it is present in fallopian tubes and endocervix. FT-06 cells did not express cytokeratin-8. End1 cells, which are derived from the endocervix, expressed cytokeratin-8 throughout the cytoplasm (Fig. 3.17, bottom). This analysis revealed that the generated FT cell lines did not originate from epithelial tissue.

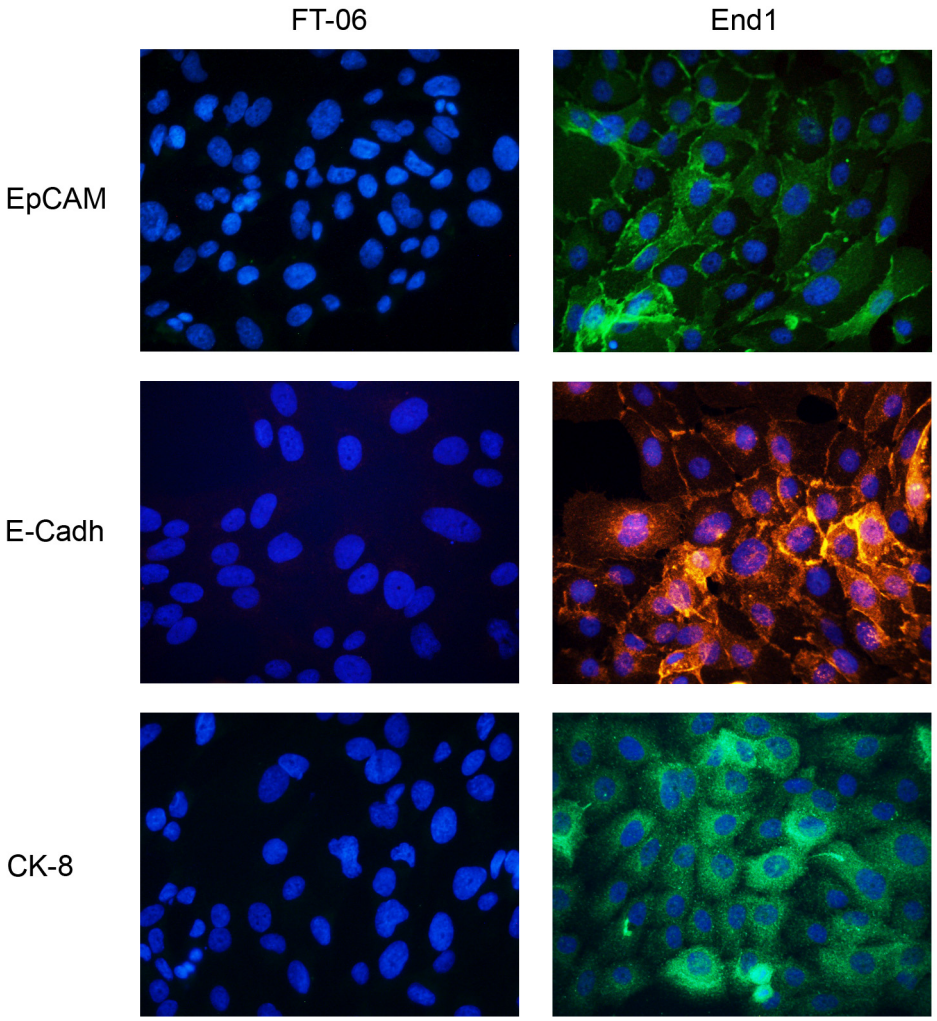


Fig. 3.17: Immortalized clonal FT cell lines do not express epithelial markers. Immunofluorescence analysis of immortalized fallopian tube cell line FT-06 carrying SV40T and Bmi1 and epithelial cell line End1 for epithelial markers. Top: Cells were stained for the epithelial cell adhesion molecule (EpCAM; green). FT-06 cells did not express EpCAM. In End1 cells, EpCAM was localized at cell borders. Middle: Staining for E-cadherin (E-Cadh, red). FT-06 cells did not express E-cadherin. In End1 cells, E-cadherin was detected at cell-cell-contacts. Bottom: Cells were stained for cytokeratin-8 (CK-8, green). FT-06 cells did not express cytokeratin-8. In End1 cells, cytokeratin-8 was localized in the cytoplasm. DNA was stained with Hoechst (blue).

To further explore from which cell type the generated FT cell lines originated, samples were analyzed for the endothelial marker platelet/endothelial cell adhesion molecule 1 (PECAM-1). Confluent fallopian tube FT-06 cells, which are shown as example, did not express PECAM-1 (Fig. 3.18). In contrast, PECAM-1 was localized at cell-cell-contacts in primary endothelial HUVEC. Note that primary HUVEC were not confluent. This experiment led to the conclusion that the obtained FT cell lines were not endothelial cells.

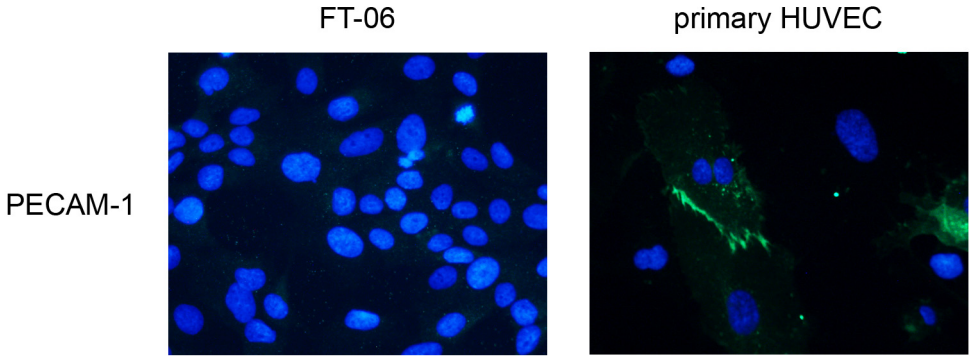


Fig. 3.18: Immortalized clonal FT cell lines are not from endothelial origin. Immunofluorescence staining of immortalized fallopian tube cell line FT-06 and primary HUVEC endothelial cells for PECAM-1 (green). FT-06 cells did not express the endothelial marker PECAM-1. Primary HUVEC expressed PECAM-1 at cell-cell-contacts between confluent cells. Absence of staining in other HUVEC can be explained by lack of confluence. DNA was stained with Hoechst (blue).

Because the generated FT cell lines turned out to be neither epithelial nor endothelial, other possible cell types were investigated. It is known that multipotent adult mesenchymal stem cells are found in the human fallopian tube and can be isolated from this tissue (Jazedje, et al., 2009). Thus, possible cell types included mesenchymal cells and stem cells, which express markers as CD44, Oct4 and N-cadherin. CD44 is a marker of mesenchymal stem cells and multipotent adult stem cells. The transcription factor Oct4 belongs to the key regulators of stem cell pluripotency. Neural cadherin (N-cadherin) is generally regarded as mesenchymal marker and is also a marker of undifferentiated mesenchymal stem cells.

To investigate expression of these cell type markers, FT cells were analyzed for expression of CD44 and Oct4 (Fig. 3.19). FT-06 cells are shown as example. CD44 was expressed in FT-06 cells at cell-borders. In addition, it was detected weakly in the cytoplasm of most cells and stronger in spots in some cells. Oct4 was observed in a patchy nuclear pattern. In addition, cells were analyzed for expression of N-cadherin. N-cadherin was expressed in FT-06 cells and localized in spots and short stretches at cell-cell-contacts (Fig. 3.19). Thus, FT-06 cells expressed both CD44 and Oct4 stem cell

markers as well as the mesenchymal marker N-Cadherin, and therefore exhibited properties of undifferentiated mesenchymal stem cell-like cells.

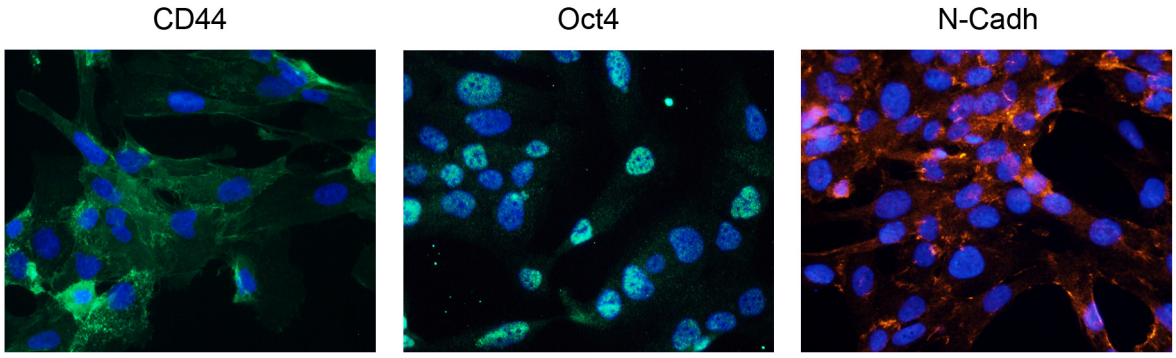


Fig. 3.19: Stem cell markers CD44 and Oct4 as well as N-cadherin are expressed in clonal FT cell line. Immunofluorescence staining of immortalized fallopian tube cell line FT-06 for CD44 (green, left), Oct4 (green, middle) or N-cadherin (N-Cadh; red). FT-06 cells expressed the stem cell marker CD44, which was detected in the cytoplasm and at cell-borders. The stem cell marker Oct4 was detected in the nucleus of FT-06 cells. N-cadherin was found in FT-06 cells in spots at cell-cell-contacts. DNA was stained with Hoechst (blue).

All cell systems analyzed so far were intended to be used for investigating pathogen-host-interactions. The human pathogenic bacterium *Chlamydia trachomatis* causes urogenital infections and naturally can infect the human fallopian tube. Therefore, *Chlamydia* infection was studied in the six best analyzed FT cell lines (FT-01 to FT-06). These cells had all been immortalized with the same combination of oncogenes (SV40T + Bmi1). Cells were infected with *C. trachomatis* serovar L2, a standard laboratory strain (Fig. 3.20). *C. trachomatis* infected all of the immortalized human fallopian tube cell lines. Chlamydial inclusions were detected throughout samples; these are the vacuoles in which bacterial replication takes place. Interestingly, *C. trachomatis* growth differed between the FT cell lines as judged by the size of the chlamydial inclusions (Fig. 3.20). The growth difference was consistent with the tissue source. FT-01 and FT-02 cells showed larger inclusions. These cells were both derived from donor tissue #1. The cell lines FT-03, FT-04 and FT-05 were derived from donor tissue #2. FT-03 and FT-04 cells carried few large inclusions as well as medium-sized inclusions. In FT-05 cells, medium-sized as well as small inclusions were found. The FT-06 cell line was derived form donor tissue #3 and mainly small inclusions were observed, with only some medium-sized ones. The analysis showed that all tested FT cell lines were readily infected by *C. trachomatis* and indicated that infection differed between the investigated FT cell lines; this seemed to be related to patient samples.

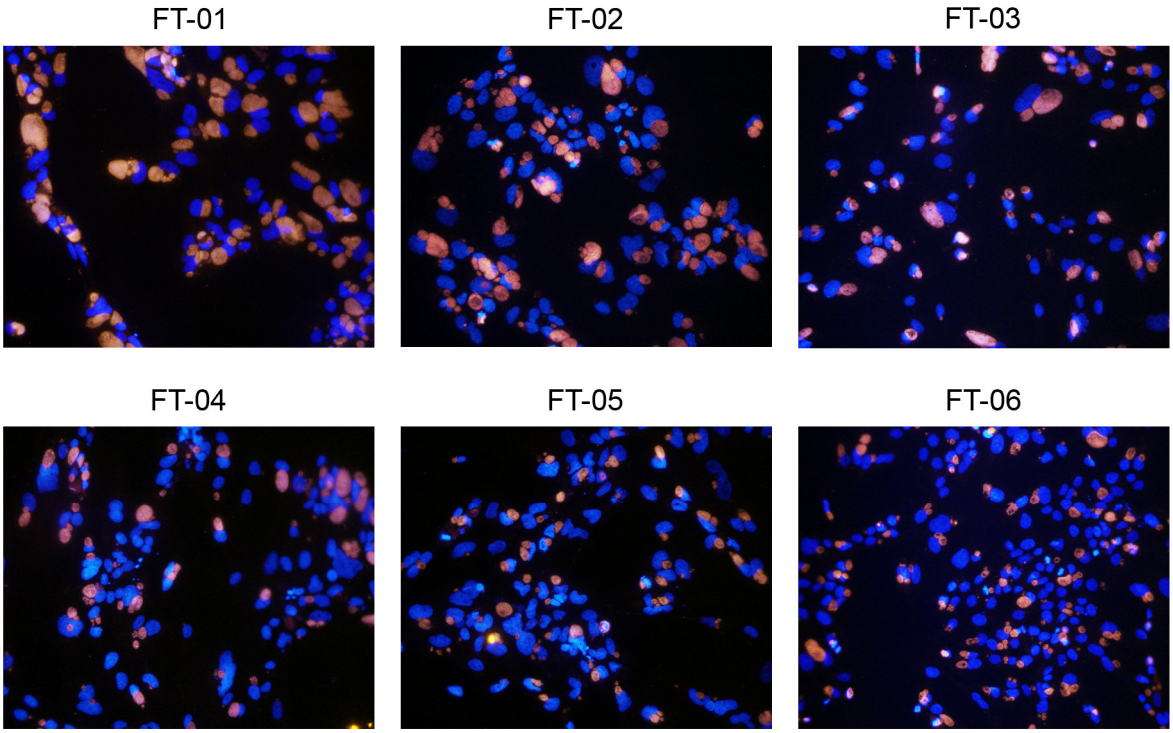


Fig. 3.20: Chlamydial inclusions differ in size between human FT cell lines. Immortalized FT cell lines were infected with *Chlamydia trachomatis* serovar L2 with MOI 3-4 for 24h. Immunofluorescence staining was performed for the chlamydial protease HtrA (red). DNA was stained with Hoechst (blue). *C. trachomatis* readily infected the immortalized human fallopian tube cell lines. Chlamydial inclusions were detected in all FT cells. FT-01 and FT-02, both derived from patient #1, showed larger inclusions. FT-03, FT-04 and FT-05 were derived from patient #2. FT-03 and FT-04 carried few large inclusions as well as medium-sized inclusions. In FT-05 cells medium and small inclusions were observed. FT-06 cell line derived form patient #3 showed mainly small inclusions with some medium-sized ones.

3.2 Part 2: Human fallopian tube *ex vivo* tissue culture as infection model for *Chlamydia trachomatis*

Urogenital and gastrointestinal pathogens first encounter the epithelial layer of target organs when entering their hosts. So far cell lines and primary cells were well established models to analyze these pathogen-host-interactions. However, pathogens do not only interact with and induce changes in single cell types. In cell culture systems the target cells are isolated. Therefore, they do not represent the *in vivo* situation present in the living host, where the cells are integrated into complex tissue structures. Structural interaction between neighbouring cells plays an important role for polarization and barrier function of epithelial cells, and various cell types build and interact within the tissue. Pathogens also often modify cellular mechanisms, which are directly dependant on or regulated by tissue homeostasis, e.g. cell cycle and apoptosis, apico-basal transport, adhesion and inflammation. Thus, pathogens can induce changes that have an impact on

the entire tissue. As consequence, these cellular mechanisms are difficult to be analyzed in cell culture.

In order to circumvent the limitations of single-cell type-cultures, we were interested to establish an *ex vivo* tissue culture model of human fallopian tubes to analyze the complex interactions of the human pathogenic bacterium *Chlamydia trachomatis* with host cells of the female genital tract. With this model, the chlamydial infection can be studied directly in the tissue context, as it occurs *in vivo* [1].

3.2.1 Establishment of human fallopian tube *ex vivo* tissue culture

For the establishment of tissue culture, healthy human fallopian tubes (FT) were obtained from the department of Obstetrics and Gynecology, Campus Virchow-Klinikum, at Charité University Hospital, Berlin, Germany, with given consent of patients. Samples were prepared as described above (see chapter 3.1.1), without removal of epithelium or digestion treatment. Pieces of FT tissue were cultured for 1-5 days.

In order to analyze the complex thick tissue, microtome sectioning was necessary to obtain samples thin enough for analysis. In order to check whether the tissue was intact after processing and the procedure was suitable, tissue sections were analyzed microscopically. Stained thin sections revealed many complete mucosal folds protruding into the lumen of the tube (Fig. 3.21A). Two layers of muscle comprise the tubal wall.

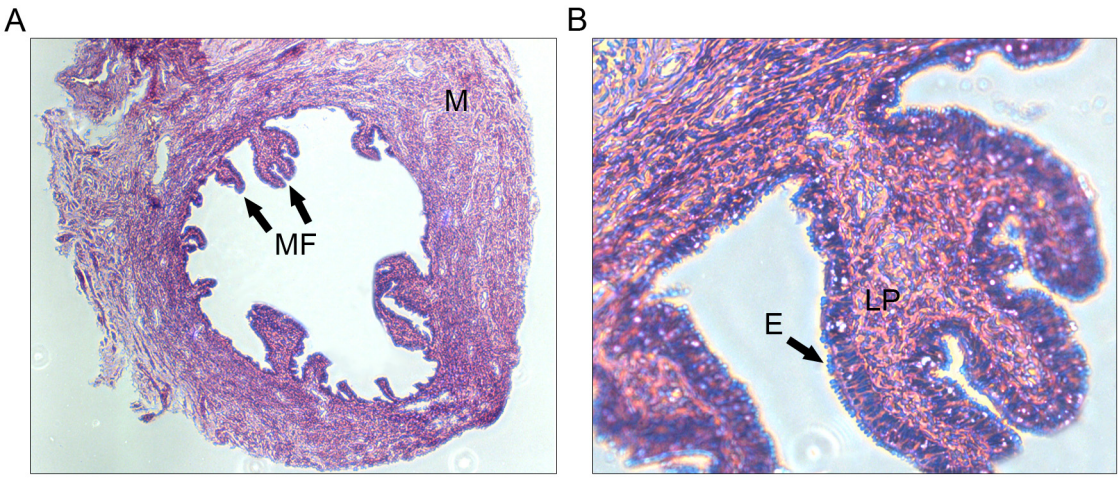


Fig. 3.21: Histology of human fallopian tube. Hematoxillin-eosin staining of human FT. A) Overview of cross-section of human FT tissue. The mucosal folds (MF, arrows) protruded into the lumen of the tube. Two layers of muscle (M) comprise the tubal wall. **B)** Enlargement of mucosal fold. The mucosa consists of the single-layer epithelium (E, arrow) and the lamina propria mucosae (LP) underneath, which is loose connective tissue. The mucosa was largely intact after culture and embedding procedures.

[1] This project was performed in cooperation with Dr. Mirjana Kessler, my direct supervisor. Predominantly, Dr. Kessler coordinated the project and helped with experimental design, I performed experiments and analyzed the data.

Higher magnification of a mucosal fold revealed that the mucosa was largely intact after culture and embedding procedures (Fig. 3.21B). The mucosa consists of the single-layer epithelium and the lamina propria mucosae underneath, which is loose connective tissue. Thus, the general tissue architecture was preserved in processed cross-sections showing that applied protocols were suitable for analysis.

Next, we wanted to analyze whether FT tissue stayed viable in *ex vivo* culture. For detailed analysis of molecular markers on a subcellular level, an optimised immunofluorescence staining protocol for paraffin-embedded tissue was applied on thin cross-sections, and samples were analyzed with high resolution confocal microscopy.

To determine functional integrity of the epithelium in the tissue culture, localization of molecular markers was analyzed. Preservation of the apico-basal axis is a major determinant of homeostasis of epithelial layers. E-cadherin is a transmembrane component of adherens junctions, thereby mediating cell-cell-adhesion. Beta-catenin is involved in connecting E-cadherin to the actin cytoskeleton. Occludin is a component of tight junctions, which are needed for epithelial barrier tightness and only are fully established in polarized epithelium. All three markers showed the localization expected in polarized epithelium. E-cadherin formed clear bands at the lateral cell membrane of epithelial cells at cell borders (Fig. 3.22A). Beta-catenin was observed at lateral cell membranes in epithelial tissue (Fig. 3.22B). In addition, occludin could be stained in small spots at the top end of lateral cell membranes (Fig. 3.22B). These data revealed that the *ex vivo* tissue culture was optimal, as the epithelium maintained its polarity and was viable for the time of culture. Further structural components of FT epithelium are cytokeratin-8, a cytoskeleton component of simple epithelia, and acetylated tubulin, which is present in cilia. Both markers were exclusively located in the epithelial layer as expected. Cytokeratin-8 demonstrated preserved tissue structure of mucosal folds with intact epithelium (Fig. 3.22C). An intact single-layer epithelium with apico-basal orientation was observed together with the lamina propria. Acetylated tubulin enabled detection of cilia at the apical side of some cells facing the lumen of the tube (Fig. 3.22D). This staining allowed discriminating between ciliated cells and the non-ciliated secretory cells of the epithelium. These observations revealed structural integrity of the epithelium after *ex vivo* tissue culture. Overall experiments revealed that tissue was in general viable for the tested period of 3-5 days in culture.

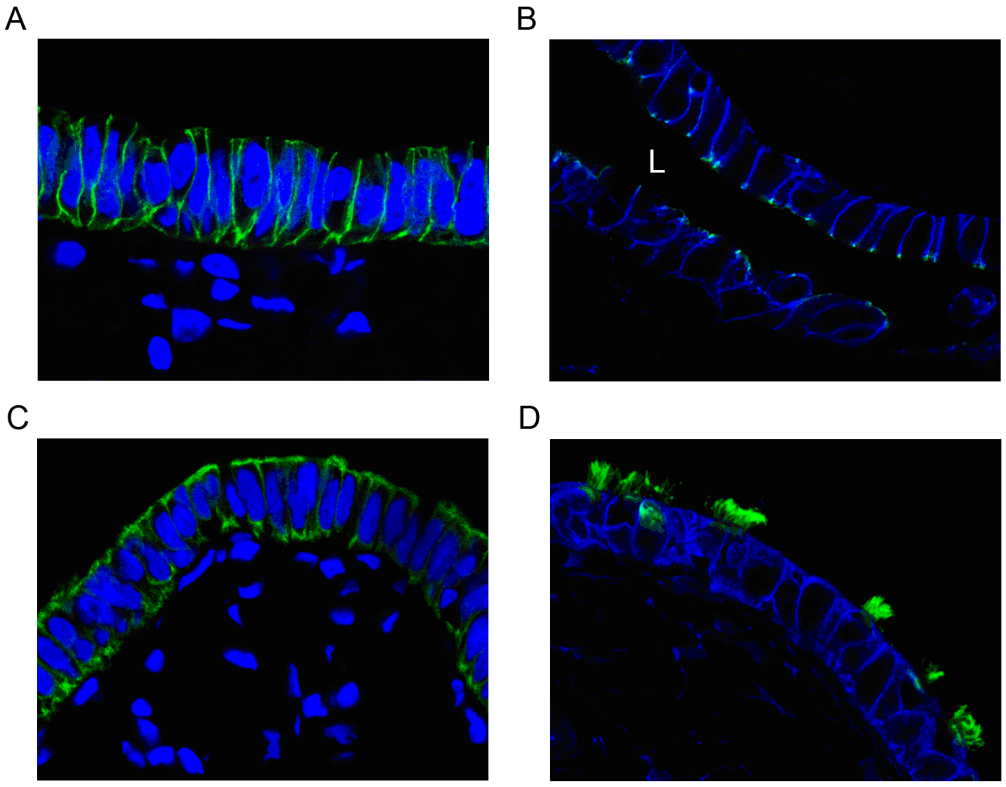


Fig. 3.22: Tissue structure, polarity and viability are preserved in culture. Immunofluorescence analysis of tissue cross-sections. Tissue cultured for 24h. Pictures display mucosal folds (as in Fig. 3.21B). **A)** Staining for E-cadherin (green), and DNA (Draq5, blue). E-cadherin formed clear bands between cells in the area of the lateral cell membrane and cell borders. **B)** Staining for occludin (green) and β-catenin (blue). Distinct spots of occludin were observed at the upper part of lateral membranes facing the lumen (L). **C)** Staining for cytokeratin-8 (green) and DNA (Draq5, blue) revealed preserved tissue structure of mucosal folds after tissue culture, embedding and staining. **D)** Staining for acetylated tubulin (green). Ciliated cells could be discriminated from non-ciliated secretory cells; β-catenin stained in blue.

3.2.2 *Chlamydia trachomatis* successfully infects human fallopian tubes *ex vivo*

Human fallopian tube tissue was used to study the infection of the human pathogenic bacteria *Chlamydia trachomatis*, which are a cause of sexually transmitted diseases. To check whether *C. trachomatis* could successfully infect the tissue, fallopian tubes were infected with bacteria and cultured *ex vivo* (Fig. 3.23A). Chlamydial inclusions, i.e. the vacuoles in which the bacteria replicate, were observed in the tissue 24h post infection (p.i.). The epithelial layer stayed intact, as polarization and cytokeratin expression of epithelial cells were preserved. The degree of infection varied locally within the tissue as well as between samples. Chlamydial serovars D and L2 both infected the tissue. For discriminating whether the bacteria replicated and differentiated inside the tissue or only invaded the epithelium, bacteria were collected from infected tissue. Therefore, medium supernatant from tissue, which had been infected for 3 days, was transferred onto Hela229 cells (Fig. 3.23B). After three days, prominent chlamydial inclusions were

observed in the cells. The presence of chlamydial inclusions demonstrated that the bacteria successfully infected the FT tissue, replicated inside the tissue and differentiated to form infectious progeny. Thus, bacteria were able to establish a replicative niche inside the tissue and complete their developmental cycle.

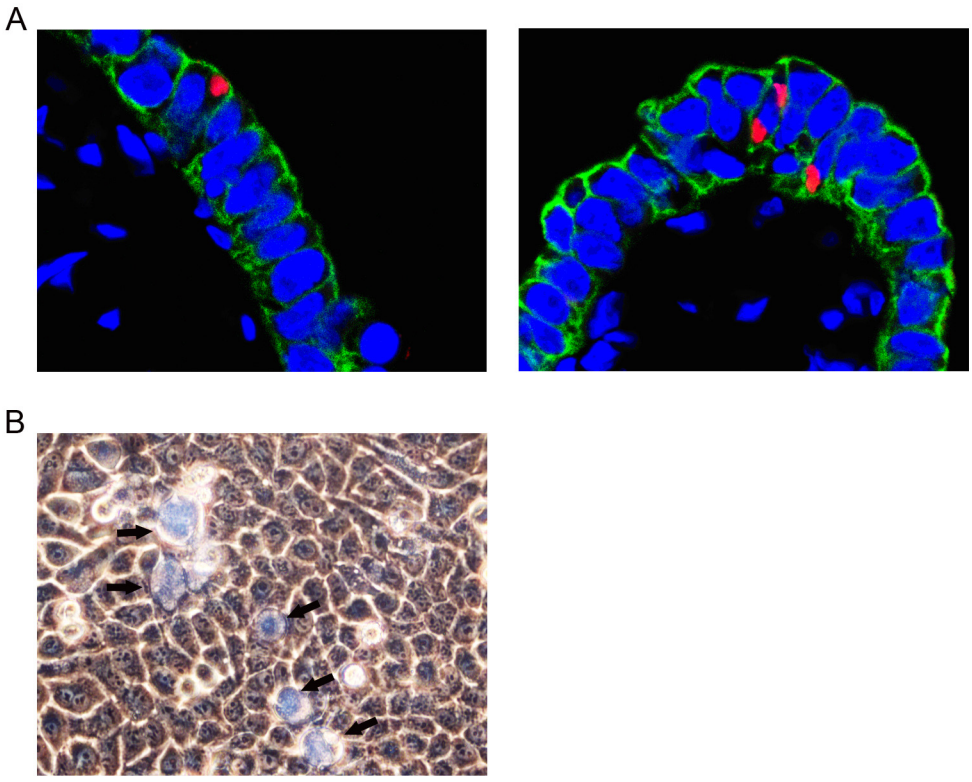


Fig. 3.23: *Chlamydia trachomatis* infects fallopian tube tissue in culture and completes bacterial developmental cycle. A) Human FT tissue was infected with *C. trachomatis* serovar D for 24h. Samples were analyzed in immunofluorescence staining for bacterial LPS (red) and cytokeratin-8 (green); DNA was stained with Draq5 (blue). *C. trachomatis* was able to infect the epithelium of tissue in culture, as bacterial inclusions (red) were observed. The degree of infection varied locally within the tissue as well as between samples. **B)** Medium supernatant from infected tissue, which had been infected for 3 days, was transferred onto Hela229 cells. 3 days p.i., large chlamydial inclusions (arrows) were observed in infected cells.

3.2.3 *C. trachomatis* infection leads to destruction of epithelium in late stages of infection cycle

In order to analyze the course of *C. trachomatis* infection in the tissue over time, we checked whether changes in the infected *ex vivo* tissue culture occurred. Therefore, FT tissue was infected with *C. trachomatis* serovar D for a time course. Samples were analyzed for the adherens junctions' component E-cadherin and *C. trachomatis* (Fig. 3.24A). In non-infected tissue, apico-basally polarized epithelium with the characteristic lateral localization of E-cadherin was observed. In early chlamydial infection, here analyzed 24h p.i., the E-cadherin staining pattern did not differ significantly from non-

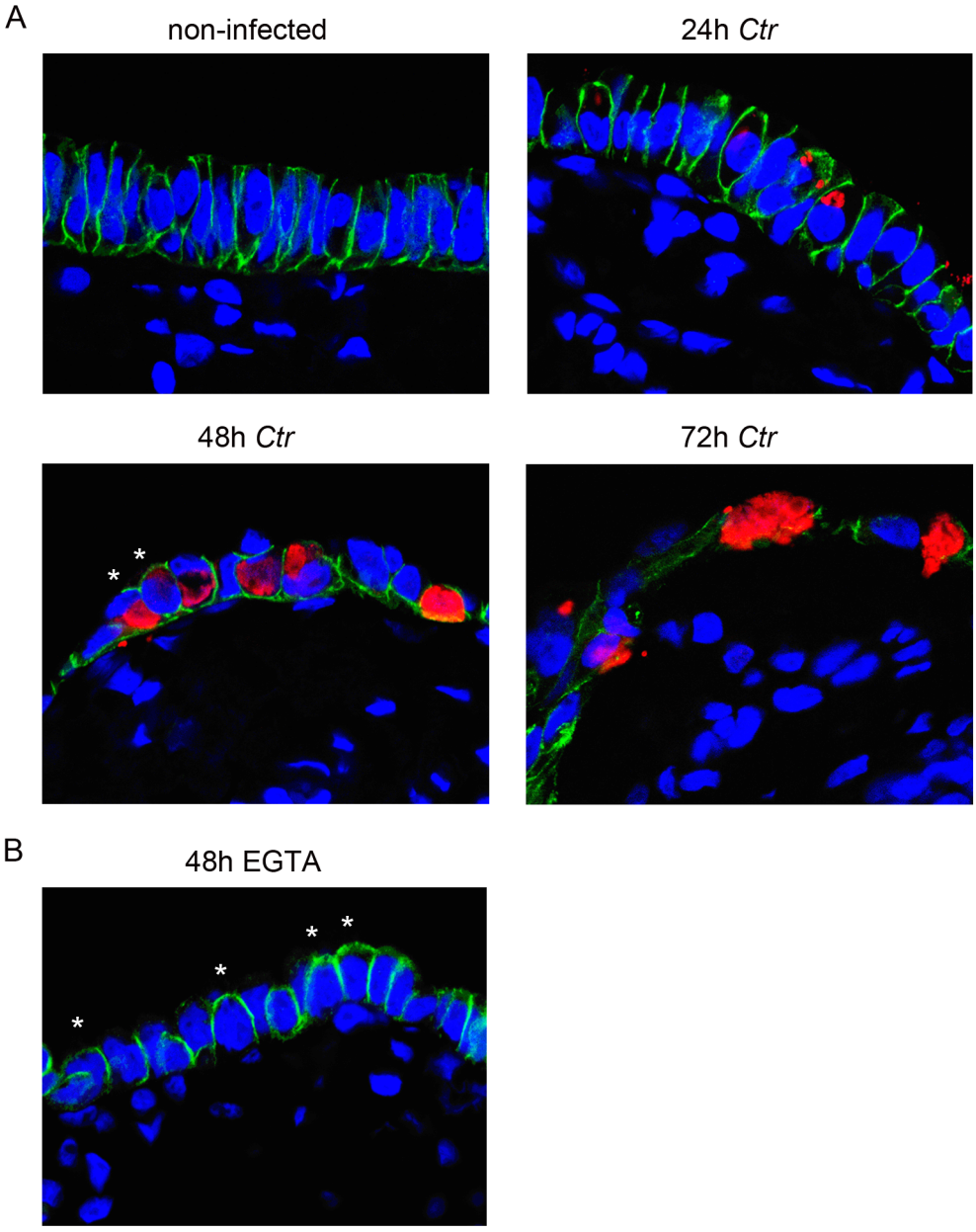


Fig. 3.24: Disruption of epithelium and loss of adhesion occur in late stages of *Chlamydia* infection. A) Human fallopian tube tissue was infected with *C. trachomatis* (*Ctr*) serovar D for 24h, 48h or 72h or cultured non-infected. Immunofluorescence analysis was performed with staining for E-cadherin (green) and *C. trachomatis* (red), DNA was stained with Draq5 (blue). In non-infected tissue, columnar epithelial cells were observed with intact lateral bands of E-cadherin. In early chlamydial infection, i.e. 24h p.i., the infected epithelium stayed intact. Both chlamydial inclusions and clear lateral E-cadherin staining were observed. 48h p.i., epithelial cells started to loose apico-basal polarity. In some cells, E-cadherin was found dislocated apically (asterisks). In late stages of chlamydial infection, 72h p.i., cell adhesion was lost and the epithelium was disrupted. Cells lost shape of polarized columnar cells. **B)** Tissue treated with EGTA (5 mM) for 48h was stained for E-cadherin (green) with immunofluorescence. Treatment with EGTA led to loss of apicobasal polarity, as E-cadherin was dislocated to the apical side (asterisks).

infected tissue, as was observed before (compare Fig. 3.23), with chlamydial inclusions inside epithelial cells. However, later in infection, significant changes in phenotype were detected within *C. trachomatis* infected mucosa; here analyzed 48h p.i.. The degree and

speed of induced rearrangements in epithelium depended on the infection rate in individual tissues. In some cells, the otherwise laterally located cell-adhesion marker E-cadherin was also found redistributed to the apical membrane. In late stages of chlamydial infection, 72h p.i., cells had lost the shape of polarized epithelium and were flattened. E-cadherin was lost in some cells, in others observed diagonally to the axis of the epithelial layer. Cell adhesion was lost and the epithelium was disrupted. Notably, within infected epithelium loss of polarity and adhesion was not restricted only to inclusion containing cells. Non infected cells in the neighbourhood also showed phenotypes consistent with loss of epithelial tissue architecture.

A similar phenotype was observed upon treatment of tissue with EGTA. EGTA is a chelating agent binding the Ca^{2+} ions required for cadherin-mediated cell-cell-contacts, and thus is a known inhibitor of adhesion. EGTA treatment led to dislocation of E-cadherin, which was also observed apically, showing loss of apico-basal polarity and the defined adhesion belt (Fig. 3.24B). This phenotype resembled the one in late chlamydial infection and thus indicated the latter to be caused by loss of adhesion. In conclusion, results showed that *C. trachomatis* disrupts epithelial integrity. This finding demonstrates the profound effect that *C. trachomatis* exerts on the homeostasis of epithelial mucosa in the fallopian tube by altering distribution of molecular markers in infected as well as non-infected cells.

3.2.4 *C. trachomatis* serovar D induces apoptosis

Chlamydia trachomatis is known to block apoptosis in cell culture. In the literature, many reports describe the inhibition of apoptosis by *C. trachomatis*. However, most of the studies used *C. trachomatis* serovar L2. This is a widely preferred laboratory strain, which causes rare lymphogranuloma venereum disease but is not a causative agent of sterility. In contrast, the *C. trachomatis* serovar D is prevalent in patients with urogenital infections, which can ascend to the upper genital tract including fallopian tubes.

For comparison of both strains, human FT tissue was infected with *C. trachomatis* serovar D or L2. Samples were analyzed for the apoptosis-marker cleaved cytokeratin-18 (Fig. 3.25). No apoptotic cells were observed in non-infected tissue cultured for 24h, and only few apoptotic cells after 72h. The infection with *C. trachomatis* serovar D induced apoptosis already 24h p.i.. Elevated levels of apoptosis were observed 72h p.i.. The apoptotic cells were observed in a wide area of the epithelial region. In contrast, no apoptotic cells were detected after infection with *C. trachomatis* serovar L2 for 24h. Only few apoptotic cells were observed 72h p.i. with numbers comparable to the basal level in non-infected tissue. Thus, infection with *C. trachomatis* serovar D – but not L2 – indeed caused apoptosis in FT tissue samples.

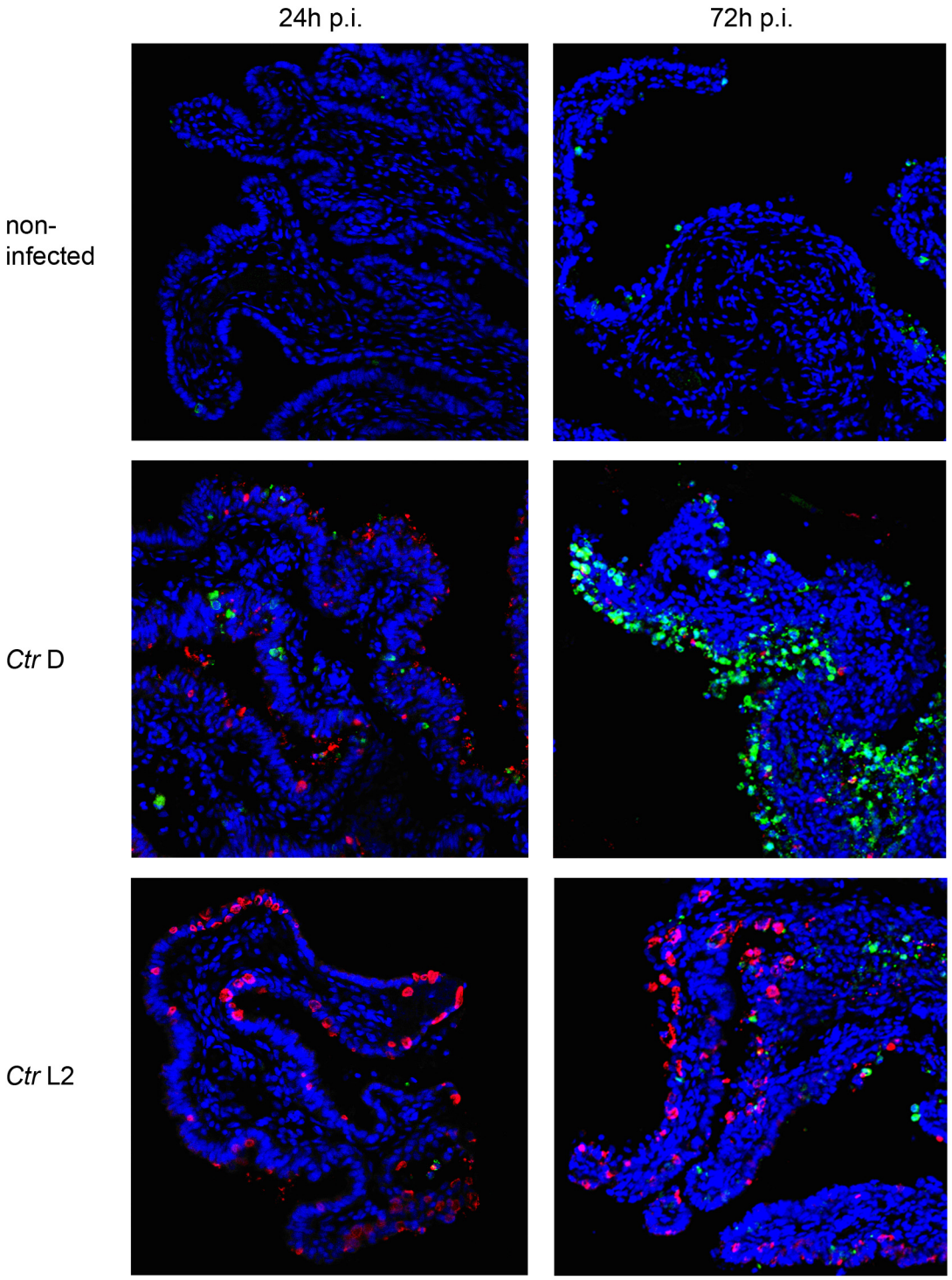


Fig. 3.25: Infection with *C. trachomatis* serovar D causes apoptosis throughout tissue. Immunofluorescence analysis of human fallopian tube tissue. Samples were infected with *C. trachomatis* serovar D (*Ctr* D) or L2 (*Ctr* L2) and analyzed 24h p.i. (left) or 72h p.i. (right) in comparison to non-infected tissue. Tissue microtome sections were stained for the apoptosis-marker cleaved cytokeratin-18 (green) and LPS (red), DNA stained with Draq5 (blue). Non-infected tissue did not contain apoptotic cells 24h p.i. and only few apoptotic cells 72h p.i.. Infection with *Ctr* D led to apoptotic cells already 24h p.i. and elevated level of apoptosis 72h p.i.. Apoptotic cells were observed in a wide area of the epithelial region. In contrast, no apoptotic cells were observed in tissue infected with *Ctr* L2 at 24h p.i., and only few apoptotic cells 72h p.i. comparable to the basal level of non-infected cells.

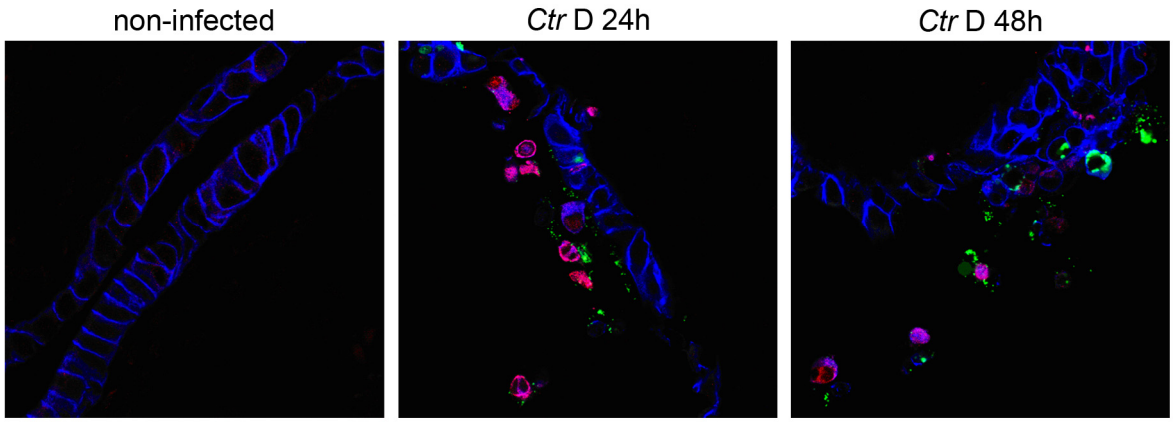


Fig. 3.26: Infection with *C. trachomatis* serovar D leads to activation of caspase-3. Immunofluorescence analysis of infected human fallopian tube tissue. Samples were infected with *C. trachomatis* serovar D (*Ctr* D) and analyzed 24h or 48h p.i., as indicated, in comparison to non-infected cells. Tissue sections were stained for the apoptosis-marker cleaved caspase-3 (red), for *C. trachomatis* (green), and E-cadherin (blue) to visualize the epithelium. Non-infected tissue did not stain either for the apoptosis-marker or bacteria. Infection with *Ctr* D induced cleavage of caspase-3. Cleaved caspase-3 was observed in tissue 24h (middle) and 48h (right) p.i..

To further verify induction of apoptosis by *C. trachomatis* serovar D, infected tissue was analyzed for an additional apoptosis marker, caspase-3. This is an effector enzyme of the apoptosis pathway. This protein is active in its cleaved form. Therefore, tissue was infected with *C. trachomatis* serovar D and analyzed for the presence of the cleaved caspase-3 (Fig. 3.26). In non-infected tissue, no caspase-3 was observed. The infection with *C. trachomatis* serovar D caused cleavage of caspase-3. 24h p.i. and 48h p.i. *Chlamydia* as well as cleaved caspase-3 were detected. The experiment led to the conclusion that *C. trachomatis* serovar D infection indeed induced apoptosis.

To confirm the phenotype of apoptosis induction, experiments were conducted in cell culture. Cell culture models raise more possibilities for manipulation of cells and for controls. As a positive control for the induction of apoptosis, the apoptosis-inducer staurosporin was included. Hela05 cells were infected with *C. trachomatis* serovar D or L2 or treated with staurosporin. After 24h, non-infected cells showed normal cell shape and viability of completely untreated cells (Fig. 3.27). In cells treated with staurosporin signs of apoptosis were observed as expected: cells with formation of apoptotic vesicles and cell shrinkage. In samples infected with *C. trachomatis* serovar D increased levels of cell death were observed, cells were elongated and accumulated vesicles. In contrast, cells infected with *C. trachomatis* serovar L2 did not reveal any signs of apoptosis but normal cell shape with typical inclusions.
In addition, these samples were analyzed for cleaved cytokeratin-18 (CK-18) (Fig. 3.28). No signal was detected in non-infected cells for cleaved CK-18, as expected. In cells

treated with the apoptosis-inducer staurosporin a strong signal for cleaved CK-18 was observed in small dots. Infection with *C. trachomatis* serovar D led to staining of cleaved CK-18, too, in a pattern comparable to staurosporin-treated cells. In contrast, when cells were infected with *C. trachomatis* serovar L2, bacterial inclusions were detected but no signal for cleaved CK-18. Because staining of the apoptosis-marker CK-18 occurred in the same arrangement as in the positive control, the experiment confirmed the finding of apoptosis-induction by *C. trachomatis* serovar D in the cell culture model.

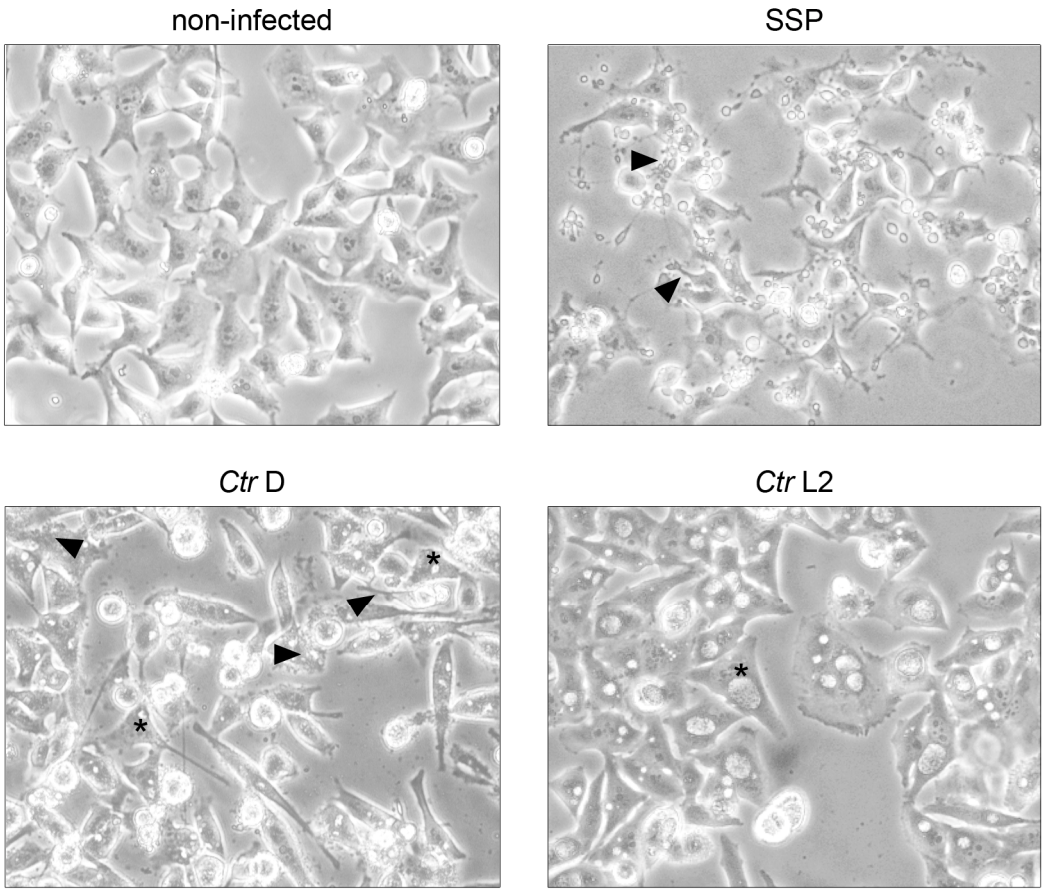


Fig. 3.27: Increased cell death is observed in cell cultures after infection with *C. trachomatis* serovar D. Phase contrast pictures. Hela05 cells were infected with *C. trachomatis* serovar D (*Ctr* D) or L2 (*Ctr* L2) with MOI 3 or treated with the apoptosis-inducer staurosporin (SSP) at 1 µM. 24h p.i., non-infected Hela05 cells, which were completely untreated, had normal cell shape and viability. SSP-treated cells showed signs of apoptosis, e.g. apoptotic vesicles and cell shrinkage (arrowheads). After infection with *Ctr* D, cell death increased. Cells were elongated and accumulated vesicles (arrowheads). Examples of chlamydial inclusions are marked by asterisks. In contrast, cells infected with *Ctr* L2 did not show any signs of apoptosis but normal cell shape with typical inclusions (asterisk).

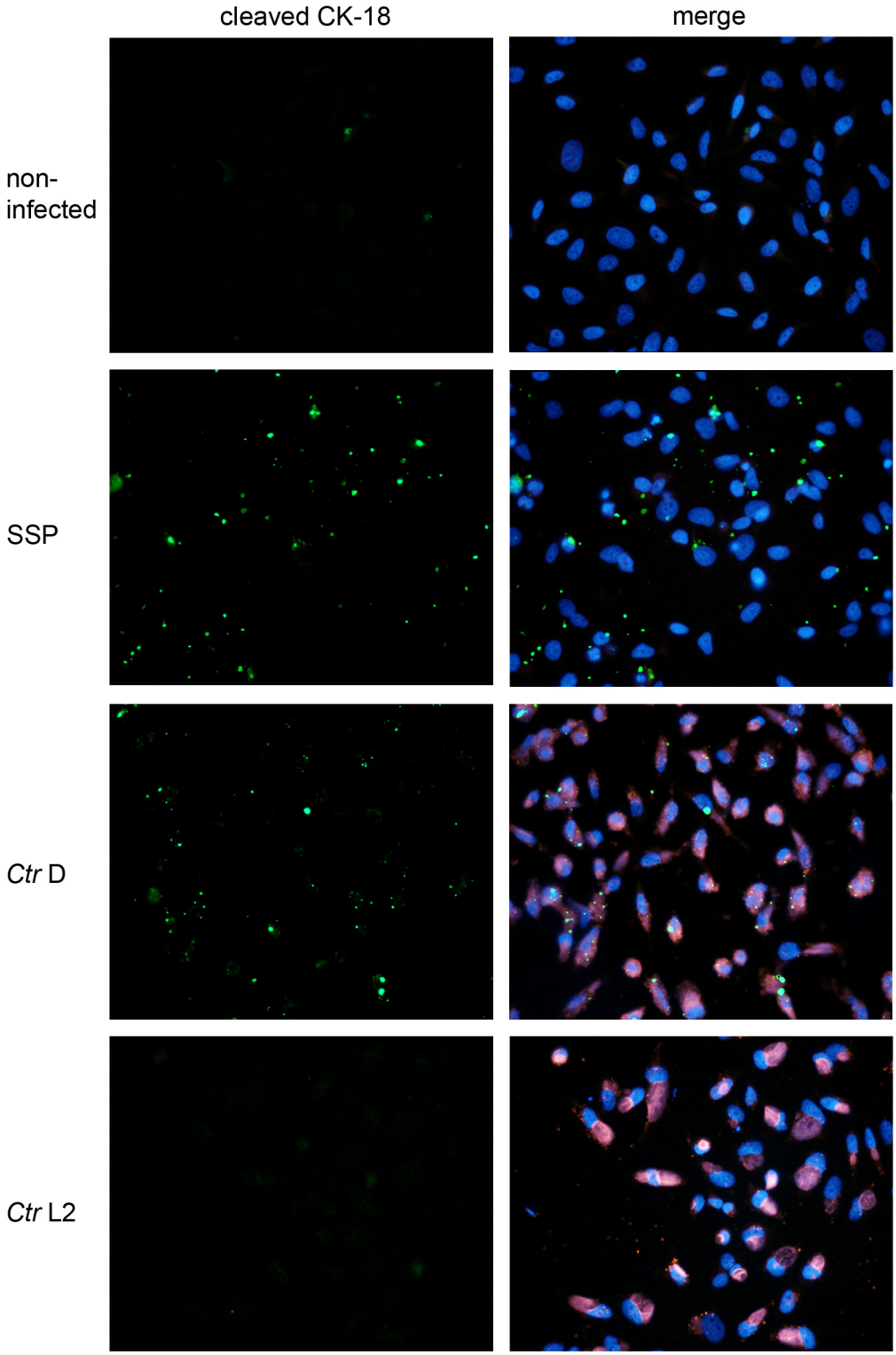


Fig. 3.28: Apoptosis induction by *C. trachomatis* serovar D is confirmed in cell culture. Immunofluorescence analysis. Hela05 cells were infected with *C. trachomatis* serovar D (*Ctr* D) or L2 (*Ctr* L2) with MOI 3, or treated with the apoptosis-inducer staurosporin (SSP, positive control) 1 µM for 24h. Samples were stained for apoptosis-marker cleaved cytokeratin-18 (cleaved CK-18; green) and bacterial LPS (red), DNA stained with Hoechst (blue). Non-infected cells did not stain for cleaved CK-18. SSP-treated cells showed strong staining of cleaved CK-18 in small dots. Infection with *Ctr* D led to cleaved CK-18-staining, too, as sign of apoptosis. In contrast, cells infected with *Ctr* L2 were negative for cleaved CK-18.

4 Discussion

Cell culture systems have long been used to study the interaction between pathogens and their living host cells. These model systems usually involve cancer-derived cell lines or immortalized cells lines of a single cell type, and due to their reduced complexity they are convenient for the analysis of single factors in pathogen-host interactions. However, these cells do not represent the condition of primary cells, as they e.g. possess infinite life span and often lack contact inhibition of growth. Additionally, in cancer-derived cell lines, the extent of changes resulting from carcinogenic transformation is unknown, especially the number of affected genes and their impact. Therefore, primary cells are used to exclude possible effects of these undefined changes. The major problems of primary cells are, however, that their proliferation capacity is usually limited and acquisition may be difficult. This complicates the direct and routine work with primary cells. In addition, all of these model systems differ from the infection situation *in vivo*, where multiple cell types interact and the cells are embedded in the complex tissue structure. Especially the barrier function of epithelial cells requires polarization and structural interaction between neighbouring cells, which are not present in normal cell culture systems.

Therefore, new primary tissue based model systems are needed to better mimic this complex situation. In this study two approaches were taken to circumvent the limitations of traditionally used cell culture systems. The aim of this study was to establish new models on the basis of (1) isolated reversibly immortalized human primary cells, and (2) intact human *ex vivo* tissue culture to analyze infections with the human pathogenic bacterium *Chlamydia trachomatis*. Human fallopian tubes were used for both studies, as *Chlamydia trachomatis* urogenital infections cause inflammation of fallopian tubes, and may lead to pelvic inflammatory disease or tissue scarring followed by occlusion of the tubes. The detailed mechanisms of pathogenesis are still not completely understood. Therefore, it is important to resolve the interaction between chlamydial pathogens and their hosts further.

4.1 Immortalization of human primary cells

Primary human umbilical vein endothelial cells (HUVEC) and primary human fallopian tube (FT) cells were successfully immortalized in this study. Expression of the transduced immortalizing oncogenes could be detected within the cells. Later on, the immortalized HUVEC were subjected to re-mortalization, i.e. the excision of oncogenes, and immortalizing constructs were shown to be excised efficiently; however, cells arrested growth. The analysis of the immortalized FT cells revealed expression of markers present in undifferentiated cell types, such as stem cells, and acquired chromosomal changes.

4.1.1 Isolation of human primary endothelial HUVEC and FT epithelial cells

In this study, protocols for the isolation and culture of human primary fallopian tube cells were established in our group, which were adapted from different published protocols. Various differing methods and descriptions can be found in literature for isolation of FT cells from human and animal tissue, including (1) mechanical disruption and enzymatic digestion, (2) uncoated and coated culture vessels as well as (3) serum-containing and serum-free media (Levanon, et al., 2010; Okada, et al., 2005; Ando, et al., 2000; Saridogan, et al., 1997; Takeuchi, et al., 1991; Henriksen, et al., 1990).

As no uniform procedure was provided in the literature, different isolation strategies were tested in this study (mainly adapted from Ando, et al., 2000; Saridogan, et al., 1997). The most successful approach included mild mechanical disruption by carefully taking small pieces of tissue of the inner mucosal folds. Culture of these explants resulted in outgrowth of primary cells from the tissue rim. Various protocols based on enzymatic digestion were tested, too, using different digestion enzymes and incubation times, and yielded differently successful results. All of these protocols are described for the isolation of epithelial cells, but all will lead to mixed populations of primary cells, not to pure cultures.

In order to optimize further parameters, coating of culture vessels with collagen and gelatine was performed as described in other protocols. However, coating did not improve the procedures, therefore the isolation protocols mainly adapted here use uncoated culture vessels. Additionally, published protocols include various media, containing serum or no serum, defined growth hormones or total gland extracts with mixed hormones. In this study, usually medium containing fetal calf serum was used, as done for routine cell culture and as described in the adapted protocols for FT epithelial cells and assisted reproduction techniques. Neither addition of epidermal growth factor (EGF) to the medium, nor usage of serum-free media containing either extracts from bovine pituitary gland or defined supplements, generally enhanced cell yield and survival in this study.

In total, primary material derived from 26 different patients, each time including one to two tubal segments, were subjected to isolation protocols during my studies. Epithelial cells could be isolated from the human FT, as cells within mixed primary isolates were positive for the epithelial cytoskeleton protein cytokeratin-8 or the epithelial cell adhesion molecule EpCAM. Taken together, primary cells could be successfully isolated and cultured with the established protocols, and mixed populations included FT epithelial cells.

As the single-layer FT epithelium is very thin and the underlying lamina propria contains fibroblasts, a main problem encountered during epithelial cell isolation is separation from fibroblasts. Keratinocyte serum-free medium (K-SFM) is optimized for keratinocytes and

cervical epithelial cells and is developed to suppress fibroblast growth (http://tools.invitrogen.com/content/sfs/brochures/332-032442_SFMBrochure.pdf). In this study, application of K-SFM did not change the obtained primary cultures compared to the other media used. This indicates that the obtained primary isolates did not contain large fractions of fibroblasts.

The usage of laser microdissection is described in the literature as another method to separate the epithelial cell layer from surrounding tissue (Lotz, et al., 2006). However, the procedure was performed with thin sections of snap-frozen intestinal tissue. Thus, the protocol would not be suitable to recover and culture cells, as the tissue is not alive any more.

With the protocol for isolation of primary HUVEC, which was available before and routinely used in the laboratory, apparently nearly pure cell populations were obtained. However, endothelial origin of primary cells was not tested before. Therefore, in this study isolated primary HUVEC were stained by immunofluorescence for the endothelial marker platelet/endothelial cell adhesion molecule-1 (PECAM-1; CD31). Primary HUVEC were positive for PECAM-1, confirming endothelial cell origin and assumed purity of isolates.

4.1.2 Successful oncogene excision in TMNK-1 cells

In order to establish an infection model with reversibly immortalized human cells, the pre-conditions for this method were tested at the beginning of this study. This included assessment of the technical aspects of reversion by oncogene excision. The recombinase Cre was used for oncogene excision, as it was already shown in 1996 that Cre is able to excise efficiently the constructs introduced into the host cell's genome, while the recombinase FLP is not (Westerman and Leboulch, 1996). The present study demonstrated efficient gene excision (re-mortalization) in the control cell line TMNK-1 by lentivirally transduced Cre-recombinase verifying the general functionality of the used system. TMNK-1 cells carry hTERT- and SV40T-constructs flanked by lox-sites, which can be excised by Cre-recombinase (Matsumura, et al., 2004). Efficient gene excision was demonstrated in FACS analysis leading to excised constructs in 66 % of cells to over 95 % at high MOI. Cell viability of re-mortalized cells was preserved after the procedure, as seen in growing cell culture.

Thus, the result of this study - the efficient Cre-mediated gene excision in TMNK-1 cells -, is consistent with the finding of Matsumura, et al. (2004), who used a different method of transferring Cre. Purified Cre-fusion proteins were directly transfected into immortalized cells in their experiments and yielded up to 60 % cells with excised constructs in a dose-dependent manner. In their study, reverted TMNK-1 cells slowly lost proliferative capability, which was monitored until three weeks after beginning of Cre treatment. In the

present study, growth of reverted cells slowed down, but cells were still able to grow confluent. Cells were monitored until 13 days after beginning of Cre treatment. Thus, re-mortalization was feasible and the procedure worked in our hands with the altered protocol including lentiviral transduction of Cre.

Besides the direct transfection of purified Cre-fusion proteins into the target cells (Matsumura, et al., 2004), various methods including the usage of viral vectors are described for gene transfer. Due to different widely used viral vectors, different protocols for Cre-mediated excision of oncogenes are described in literature, including adenoviral vectors (Salmon, et al., 2000) or lentiviral vectors (Nguyen, et al., 2005; Cudré-Mauroux, et al., 2003).

4.1.3 Immortalization and oncogene excision in the endothelial model system

Successful immortalization was achieved in HUVEC with combinations of each two of the oncoproteins hTERT, SV40T and Bmi1 resulting in unlimited cell growth. Cells were monitored up to passage 50. Cell lines were also obtained with SV40T alone. Oncogene expression was confirmed by real time RT-PCR and the immortalized cells retained endothelial phenotype, as they still expressed the endothelial marker PECAM-1. In contrast to the immortalized HUVEC, HUVEC transduced with only hTERT- or Bmi1-construct did not grow continuously, growth was arrested and cells finally died. Thus, it was concluded that hTERT or Bmi1 alone were not sufficient for immortalization and the cells entered senescence. Insufficiency of hTERT or Bmi1 alone for immortalization was observed by Cudré-Mauroux, et al. (2003) as well. Taken together, the successful immortalization of primary HUVEC in this study using lentiviral gene transfer showed that the system can be applied for the immortalization of primary cells.

PECAM-1 was shown to be a stable endothelial marker in this study, which is in agreement with other groups who reported maintenance of PECAM-1 expression. Oostingh, et al. (2007) compared primary HUVEC and human dermal microvascular endothelial cells with four immortalized endothelial cell lines from brain, placenta or dermis, and reported loss of two endothelial adhesion molecules (E-selectin and VCAM-1) in the immortalized cells. At the same time, PECAM-1 (CD31) was still expressed in all but brain immortalized endothelial cells and expression was comparable to the primary cells. Stability of endothelial marker expression including PECAM-1 was also reported for hTERT- and SV40T-immortalized HUVEC after over 100 population doublings (Tentori, et al., 2005) and HUVEC immortalized with human papillomavirus 16 E6/E7 in approximately passage 30 (Fontijn, et al., 1995). Additionally, a reversibly immortalized HUVEC cell line was achieved by Noguchi, et al. (2002) via retroviral transfer of SV40T, and cells retained expression of endothelial markers.

In contrast, van Leeuwen, et al. (2000) reported loss of endothelial markers such as PECAM-1 and von Willebrand factor in HUVEC immortalized with SV40T. PECAM-1 expression declined with continuous cell growth (measured in cell passages 13-31) and was not detected in cells at high passages (measured up to passage 89). In the present study, cells were observed for a shorter time period up to passage 50. Therefore, changes in marker expression in later cultures can not be excluded.

For re-mortalization, oncogene excision via the recombinase Cre was achieved in immortalized HUVEC in this study following the protocol successfully tested with TMNK-1 control cells. However, soon after Cre treatment the reverted HUVEC arrested growth completely. The reasons for this permanent growth arrest were so far unclear as the principal usability of lentivirally transduced Cre-recombinase was shown in this study for TMNK-1 cells (see above) and efficient Cre-transduction was also reported by other groups using retroviruses or adenoviruses (Kowolik, et al., 2004; Salmon, et al., 2000). It was expected that the re-mortalized HUVEC would rather behave like the Cre-treated TMNK-1 cells in this study, which slowed down the cell growth but did not completely arrest it. Therefore the observed complete growth arrest of the re-mortalized HUVEC was unexpected.

In contrast to our study, Noguchi, et al. (2002) were able to perform successful reversion (i.e. re-mortalization) of immortalized HUVEC to a pre-transduced state, in which cells did not enter senescence directly. Their study differs from our methods, as the authors used adenovirus-mediated transfer of the recombinase Cre with MOI 5. Reverted cells were selected by using the antibiotic G418, as reverted cells in their system expressed the neomycin resistance gene. In addition, growth characteristics after oncogene excision were influenced by the time span the cells had been cultured. Cells with lower population doublings maintained cell proliferation after reversion.

Although Noguchi, et al. (2002) state that the immortalized endothelial cells can be reverted without any problems, Cre-induced growth arrest was reported by other groups. When the TMNK-1 cell line was originally published, a slow loss of cell proliferation after re-mortalization was reported (Matsumura, et al., 2004). A Cre-induced growth arrest was also observed by Salmon, et al. (2000) in liver endothelial cells already two to four days after Cre-treatment. The authors concluded that cell division of the immortalized cell line still strictly depended on the presence of the immortalizing genes. Other studies also reported growth arrest of cells after addition of Cre recombinase to reversibly immortalized cells (Narushima, et al., 2005; Cudré-Mauroux, et al., 2003; Kobayashi, et al., 2000). In our study, the cells seem to be still dependent on the oncogenes as well.

Notably, in this study HUVEC immortalized with hTERT + Bmi1 partially survived treatment with ganciclovir. One possibility is that the cells were less sensitive to ganciclovir than the other immortalized HUVEC, and that Cre-mediated oncogene excision was not efficient in HUVEC with hTERT + Bmi1. Alternatively, primary cells might be present in between immortalized cells. Primary HUVEC were shown not to be affected by ganciclovir. However, it is not likely that primary cells were still present in these long-term cultures.

Silver and Livingston (2001) showed that continuous expression of Cre-recombinase led to decreased growth, cytotoxicity and chromosomal aberrations in the transduced cells. These negative effects were avoided by using a self-excisable Cre-construct, which is flanked by lox-sites. Thus, the amount and duration of Cre expression were limited. As a self-excisable Cre-construct was used in the present study, this cytotoxicity effect could be excluded.

Despite several studies describing successful and feasible re-mortalization of cells in culture, there are only few reports about direct applications of reversibly immortalized cell lines after reversion back to a pre-transduced state. Kobayashi, et al. (2000) transplanted reverted human hepatocytes into rats and successfully prevented acute liver failure. Transplantation of reverted insulin-producing hepatocytes into pigs and of human pancreatic β-cells into mice resulted in control of diabetis (Narushima, et al., 2005; Okitsu, et al., 2004).

4.1.4 Primary human fallopian tube cells were successfully immortalized

For the isolation of primary human fallopian tube cells, tissues from 26 different patients were obtained, each time including one to two tubal segments. In total, mixed primary cell populations of 18 FT tissue pieces (derived from 12 different patients) could be used in 10 independent immortalization experiments. Combinations of the oncoproteins hTERT, SV40T and Bmi1 as well as single oncoproteins were used. FT cells showed stress vacuoles and fibres after transduction with oncogenes. The mixed primary cell populations used for immortalization contained epithelial cells, but were not pure cultures. As pure isolation of epithelial cells was difficult, clonal expansion was performed via flow cytometry single cell sort or cloning rings. In most cases, single cells did not grow and died, when cells were singled to obtain clones. The survival rate did not increase when culture vessels were coated. Altogether, 21 FT cell clones were obtained. The transduced primary cells had proliferated only in the presence of SV40T, and all obtained FT cell clones were immortalized with SV40T and Bmi1. Oncogene expression was verified in FT cell clones.

The oncoprotein hTERT alone was not sufficient for immortalization (as was demonstrated in this study already with HUVEC). It remains unclear why no FT cells immortalized with hTERT plus SV40T or Bmi1 were obtained in the present study. A defective hTERT construct can be excluded, as functionality of the hTERT construct was shown in HUVEC (by detection of *HTERT* expression and cell immortalization via hTERT in combination with SV40T or Bmi1). Immortalization of human fallopian tube epithelial cells was achieved by other groups using HPV16 E6/E7, TERT plus SV40T or SV40T alone (Lee, et al., 2010; Lee, et al., 2001; Ando, et al., 2000). Until now, there are no reports of reversibly immortalized human fallopian tube epithelial cells.

Reversible immortalization was implemented into this study to have an option to revert the cells back to a primary-like state. When immortalized cells were reverted, it was assumed that they adjusted growth properties to the ones of primary cells. In the initial experiments, reversion of TMNK-1 cells could be shown to work efficiently, and cells retained a lower proliferation capacity. However, experiments with immortalized HUVEC in this study and reports by other groups showed that in many cases reverted cells stopped growth very soon after Cre-treatment (see above). Based on the findings from these experiments it was concluded that cells can be re-mortalized but undergo cell cycle arrest. The procedure of Cre-treatment and selection of reverted cells takes up to two weeks. Therefore, the cells had no proliferation potential any more by the time they could be applied to other experiments. The major problem here is that these follow-up studies themselves can take several days or longer.
Strikingly, García-Escudero, et al. (2010) reported that only ensheathing glia cells reversibly immortalized with Bmi1 and hTERT still proliferated after re-mortalization, when they compared different combinations of SV40T, hTERT and Bmi1. None of the other cells could revert back to this primary cell replicative state.
In addition, it is known from literature that epigenetic changes can occur after introduction of oncogenes. In case of Bmi1, histone modifications are influenced. Bmi1 is involved in gene silencing by histone H3 methylation (Agherbi, et al., 2009; Bracken, et al., 2007). Additionally, Bmi1-dependent histone 2A ubiquitylation was described (Kallin, et al., 2009).
Due to all these reasons, re-mortalization could not be extensively studied in the FT cell lines.

All 21 FT cell clones generated in this study were analyzed for the expression of at least one or all three epithelial markers (EpCAM, E-cadherin, and cytokeratin-8). None of the cell lines expressed epithelial markers and thus the cells were not regarded as epithelial

cells. In addition, all cells tested were negative for the endothelial marker PECAM-1, so endothelial origin of immortalized cells was excluded. In the literature the epithelial cells were distinguished from fibroblasts by many authors via their cell shapes (Ando, et al., 2000; Saridogan, et al., 1997; Takeuchi, et al., 1991; Henriksen, et al., 1990). They described the primary epithelial cells in FT isolates as irregular, polygonal and slightly elongated. In this study, the obtained FT clones differed in their cell shape. While some grew irregularly round and tightly confluent, others were elongated and more spindle-like.
When the cells were grown on permeable filter supports to induce polarized growth, the trans-epithelial electric resistance (TEER) increased, albeit absolute values were low. A rising TEER usually indicates closure of gaps between the cells and thus between both sides of the filter, indicating barrier formation by tight junctions. However, EM analysis revealed that no FT cell line was single-layered or polarized with an apico-basal axis including tight junctions and apico-basally oriented nuclei. The cells did not show properties of polarized epithelial or endothelial cells. The low rise in TEER values might have been caused by blockage of filter pores by multiple cell layers.

4.1.5 Immortalized FT cells express stem cell markers

Interestingly, in this study immortalized FT cells were shown to express the stem cell markers CD44 and Oct4 as well as the mesenchymal and stem cell marker N-cadherin. CD44 is a glycoprotein localized on cell surfaces, which is involved in cell-cell-adhesion and cell migration (Aruffo, et al., 1990; Stoolman, 1989). In addition, CD44 is a marker of mesenchymal stem cells and multipotent adult stem cells. For example, CD44 is expressed in prostate epithelial stem cells and breast stem cells (Jazedje, et al., 2009; Zuba-Surma, et al., 2009; Zenzmaier, et al., 2008; Mani, et al., 2008). As it is known that CD44 is expressed in human fallopian tube stem cells (Jazedje, et al., 2009), the cells isolated in this study could indeed be stem cells.
The transcription factor Oct4, also known as POU5F1, is one of the key regulators of stem cell pluripotency and is required to keep embryonic stem cells in their undifferentiated state. The transcription factor is described not to be active in normal somatic cells (Nichols, et al., 1998; Pei, 2009; Marikawa, et al., 2005). However, Oct4 expression was reported in very small embryonic-like stem cells and ovarian cancer stem cells (Zuba-Surma, et al., 2009; Peng, et al., 2010). Thus, the observed Oct4-positive FT cells from this study have a stem cell-like phenotype.
N-cadherin (neural cadherin or cadherin 2) is expressed in neural and non-neural cells such as myocardiac cells and hematopoietic progenitor cells (Takeichi, 2007; Wein, et al., 2010; Hatta, et al., 1987). N-cadherin is also a marker of undifferentiated mesenchymal stem cells, which can differentiate to form cells of bone, fat tissue and muscle (Shin, et

al., 2000; Mbalaviele, et al., 2006). It is known that multipotent adult mesenchymal stem cells are found in the human FT. These cells can undergo differentiation to bone, fat, cartilage, and muscle cells (Jazedje, et al., 2009). The last authors did not analyze N-cadherin expression, but these reports support the conclusion that maybe stem cell-like FT cells were isolated in this study. The epithelial cells might have been too sensitive and maybe did not grow long enough to obtain clonal cultures, so that stem cell-like cells expanded.

In addition to the expression of CD44, Oct4 and N-cadherin in stem cells, their expression is also described in other cell types. For instance, CD44 is observed in cancer-initiating cells that play a role in tumor formation and Oct4 is detected in stem-cell-like cells found in epithelial ovarian cancer (Marhaba, et al., 2008; Peng, et al., 2010). N-Cadherin is often generally regarded as mesenchymal marker.

Loss of epithelial markers and gain of mesenchymal and stem cell properties are characteristics known from epithelial to mesenchymal transition (EMT). EMT is a process in which epithelial cells convert to a mesenchymal phenotype, and it is involved e.g. in embryonic development, tumor invasion and metastasis. EMT is characterized by loss of E-cadherin with shift to N-cadherin and expression of vimentin. In addition, EMT is connected to stem cell properties such as promoted cell survival and expression of CD44 or Oct4 (Thiery, et al., 2009; Mani, et al., 2008). EMT could have influenced cell marker expression of the isolated and immortalized FT cells. Apart from the possibility that original stem cells were isolated and transduced in this study, it is thus conceivable as well that epithelial cells were isolated, which then lost the epithelial phenotype.

It is also known from literature that markers present in primary cells can get lost in cell culture. Comer, et al. (1998) reported that epithelial FT cells adopted an immature secretory-like phenotype *in vitro*. This was reversible, as hormones could stimulate cells to differentiate and form cilia again. The degree of marker loss may vary.

The studies discussed here allow two alternative interpretations of the presented results: (1) an undifferentiated stem cell-like cell type was isolated and immortalized in this study; or (2) epithelial cells were isolated but lost their specific markers in *in vitro* culture and maybe went through EMT.

4.1.6 Immortalized fallopian tube cells acquired chromosomal changes

All FT cell lines obtained in this study were immortalized with the oncoproteins SV40T and Bmi1. Karyotype analysis revealed abnormal chromosomes in the analyzed FT cell lines. The chromosomal changes were partially extensive, including fusion chromosomes, missing chromosomes and structurally aberrant chromosomes, whose origin could not be

identified. The latter formed ring structures, contained two centromeres or consisted of fused chromosomes or only small chromosome parts.

SV40T has been used to immortalize many different cell types. Among these are numerous human epithelial and endothelial cells as well as other cells types, e.g. hepatocytes (Qiu, et al., 2006; Nguyen, et al., 2005; Kowolik et al, 2004; Maruyama, et al., 2004; Matsumura, et al., 2004; Garbe, et al., 1999). SV40T is very efficient for the induction of proliferation and immortalization of primary cells. However, in some studies the chromosomes of immortalized cells were analyzed. In these studies, SV40T led to chromosomal aberrations and partly to strong phenotypic changes in the SV40T-expressing cells (García-Escudero, et al., 2010; Cudré-Mauroux, et al., 2003).

It has long been known from literature that introduction of SV40T can induce chromosomal changes in the target cells. Ray, et al. (1992) systematically investigated SV40T-expressing cells in different stages. Soon after introduction of SV40T the cells showed distinct alterations of both chromosome number and structure, indicating chromosome breaks. Aneuploidy, i.e. an abnormal number of chromosomes, was found in nearly all cells. Analysis of the cells in later stages revealed that the SV40T induced effects were not transient but ongoing. The most frequent structural abnormalities detected were dicentric chromosomes. In contrast, few ring chromosomes and acentric fragments were observed. The SV40T-expressing cells showed chromosomal instability, as chromosome numbers varied in cultures and also changed over time. Hein, et al. (2009) reported induction of tetraploidy by SV40T.

SV40T binds to and thereby inactivates the cellular retinoblastoma (Rb) proteins. This leads to activation of transcription factors, e.g. E2F, followed by entry of host cells into the DNA replication phase. SV40T also inactivates the tumor suppressor p53 (Levine, 2009). In addition, SV40T alone was shown to be enough to induce the DNA damage response observed in SV40 infections. It disrupts genome integrity mechanisms by binding to Bub1, which functions in the mitotic spindle checkpoint. Mutated Bub1 was shown to induce aneuploidy and cancer. The elicited DNA damage response is activated via the binding of SV40T to Bub1. SV40T interaction with Bub1 might thus disturb the checkpoint and lead to aneuploidy (Cheng, et al., 2009; Hein, et al., 2009). These mechanisms could contribute to the observed aneuploidy in the immortalized FT cells. Additionally, it was reported that SV40T can bind to the MRN complex, which itself binds to double-stranded DNA breaks and usually recruits repair factors of the DNA damage response. SV40T binding to MRN can circumvent the DNA damage signalling (Cheng, et al., 2009) and thus may result in unrepaired DNA double strand breaks. The latter could explain the loss or fusion of chromosome parts, leading to the aberrant marker chromosomes observed in this study.

Cantalupo, et al. (2009) compared gene expression patterns of two different mouse cell types expressing SV40T and observed cell type specific gene regulation by SV40T in addition to the changes occurring in both cell types. This suggested that the outcome of SV40T action depends on the cell type as well.
It was shown that alterations in DNA methylation are associated with SV40T-induced carcinogenesis in a mouse model and that a *de novo* DNA methyltransferase contributes to transformation of cells in culture. Increased DNA methylation and downregulated gene expression were observed for some tumor suppressors (Soejima, et al., 2003; Komatsu, et al., 2000).
Some studies directly compared the effect of the three oncoproteins hTERT, SV40T and Bmi1 on chromosome structure in the same cellular background (García-Escudero, et al., 2010; Cudré-Mauroux, et al., 2003). In contrast to SV40T, hTERT and Bmi1 were described to maintain the normal karyotype of immortalized cells usually without chromosomal aberrations. Therefore, it is assumed that Bmi1 did not contribute to the chromosomal changes observed in the present study in cells immortalized with SV40T and Bmi1.

One possibility to circumvent karyotype changes in immortalized cells might be using alternative oncogenes. Mechanisms for the inactivation of p53 and Rb were also developed by other viruses than SV40. For example, the E6 and E7 proteins from human papilloma viruses (HPVs) high-risk strains are used for the immortalization of human primary epithelial cells (Dimri, et al., 2005; Fichorova, et al., 1997). E7 can interact with the retinoblastoma protein family. E6 targets the p53 tumor suppressor for degradation and leads to activation of the hTERT promoter and thus to telomerase activity (Moody and Laimins, 2010; Wise-Draper and Wells, 2008).
The HPV16 E6/E7 proteins are described to maintain chromosomal stability in some reports, while they seem connected to chromosomal changes in others. Fontijn, et al. (1995) reported that HUVEC immortalized with HPV16 E6/E7 retained a diploid karyotype. Deng, et al. (2008) achieved immortalization of human cervical epithelial cells with HPV16 E6/E7 with only minor structural chromosomal instability in two cell lines, while in a third cell line extensive chromosomal aberrations were observed. The successive accumulation of genomic alterations in E6/E7 immortalized cells was reported as well (Carmean, et al., 2007).

4.2 Analysis of chlamydial infection in human fallopian tube tissue culture

Ex vivo tissue culture of the human fallopian tube (FT) was successfully established and tissue was viable for 3-5 days in culture. This is the first study providing high resolution

subcellular immunofluorescence analysis of formalin-fixed paraffin embedded human fallopian tube by optimised confocal imaging.

In this study, the new tissue culture model was used to analyze the infection of *Chlamydia trachomatis*. The bacteria were shown to infect epithelial cells within fallopian tube tissue and to complete the replication cycle in tissue culture, as infectious *C. trachomatis* were recovered from medium of infected tissue. In the second half of the chlamydial infection cycle, the infection was demonstrated to disrupt the epithelium and to induce loss of cell adhesion. Furthermore, differences could be highlighted in this study between the *C. trachomatis* serovars D and L2. The serovar D was found to induce apoptosis in infected and non-infected cells in fallopian tube tissue and also in cell culture. In contrast, the serovar L2 did not induce apoptosis, as is known from literature.

4.2.1 Successful fallopian tube tissue culture and analysis by high resolution immunofluorescence confocal imaging

This study provides for the first time subcellular analysis of formalin-fixed paraffin embedded (FFPE) human fallopian tube (FT) tissue using optimised multiple immuofluorescence staining and applies this model for the analysis of chlamydial infection. The immunofluorescence protocol for high resolution confocal microscopy of FFPE tissue was developed by Robertson and colleagues (2008) to circumvent limitations in conventional immunohistochemistry. So far, analysis of FFPE tissue was commonly performed with antibodies coupled to an enzyme, e.g. peroxidase, converting a chromogenic substrate to a precipitate. Therefore, usually combinations of enzymes to detect more than one protein did not yield high quality results and could not be used for colocalization studies. Another disadvantage of this kind of detection was less resolution due to the precipitate and analysis of whole sections of several micrometers thickness in light microscopy. In contrast to the use of enzyme coupled antibodies, immunofluorescence staining was performed in cryosections instead of FFPE material, but cryosections tended to preserve the tissue structure less (Robertson, et al., 2008). Preliminary experiments at the beginning of our study had shown that cryosections of human FT tissue were distorted and torn. Robertson and colleagues (2008) demonstrated their method with human kidney and breast tissue. We investigated healthy human FT tissue and analyzed chlamydial infection therein using this optimised method.

Human fallopian tube tissue culture was established and shown to remain intact with preserved tissue structure, polarity and viability in this study. The tight junction marker occludin and the adherens junction proteins E-cadherin and β-catenin were expressed at cell junctions or laterally at cell contacts, respectively, as demonstrated by

immunofluorescence staining. Cilia were preserved in tissue culture at the apical side of ciliated cells as marked by acetylated tubulin.

The human FT tissue stayed viable in culture in this study, adhesion and polarity markers were observed as expected. Levanon, et al. (2010) obtained similar results with human FT culture staining the adhesion proteins E-cadherin and EpCAM as well as acetylated tubulin in cilia at the apical side of epithelial cells by immunohistochemistry. Other groups observed intact undisrupted epithelial surfaces with microvilli and cilia on tightly packed cells by electron microscopy of untreated tissue in culture for 1-7 days (Baczynska, et al., 2007; Hvid, et al., 2007; Cooper, et al., 1990).

So far, other studies reporting *C. trachomatis* infection of human FT in culture performed electron microscopy and conventional immunohistochemistry analysis (Baczynska, et al., 2007; Hvid, et al., 2007; Kelly, et al., 2001; Cooper, et al., 1990). Only a study by Dieterle, et al. (1998) used FFPE FT tissue from patients infected with *C. trachomatis* to perform immunofluorescence staining of chlamydial major outer-membrane protein (MOMP). However, the sample was only stained for MOMP and lacked any cellular markers to enable orientation within the tissue or to demonstrate multi-colour analysis. Furthermore, no different stages of chlamydial infection were shown. Thus, the present study is the first to demonstrate multi-channel immunofluorescence staining with detailed subcellular analysis of FFPE human FT tissue.

4.2.2 *Chlamydia trachomatis* infects and reproduces within *ex vivo* fallopian tube tissue culture

Human fallopian tube (FT) *ex vivo* tissue was infected by *C. trachomatis* in culture within this study. Chlamydial inclusions were detected exclusively in epithelial cells in the tissue at various time points of the chlamydial replication cycle. As supernatant from infected tissue gave rise to infection in cell culture, the replication cycle was completed by chlamydiae in the tissue and infectious progeny was released from the tissue.

As in our study, infection of the epithelial layer with *C. trachomatis* had been observed before in human FT tissue culture (Hvid, et al., 2007; Cooper, et al., 1990; Hutchinson, et al., 1979) confirming this as a reliable infection model. The successful replication of chlamydiae within the human FT tissue in culture is consistent with the findings of Hutchinson, et al. (1979), who were able to recover the bacteria from infected tissues and medium. Thus, chlamydial infection of human FT tissue *ex vivo* as presented here is a valid model.

4.2.3 Disruption of epithelium and loss of cell adhesion during chlamydial infection

In our study, infection of human fallopian tube (FT) tissue with *C. trachomatis* (serovars D and L2) induced disruption of the epithelial layer during later stages of the infection cycle. This was observed two to three days post infection depending on the amount of infection in individual tissues. Cell adhesion and columnar shape of polarized epithelial cells were lost.

In the literature, various changes within epithelial layers infected with *Chlamydia* are described. Rapture of single infected cells was observed 72 h post infection (p.i.) with *C. trachomatis* by Cooper, et al. (1990) in human FT tissue; however, the cytoplasmic membrane contacts to neighbouring epithelial cells appeared intact. The raptured cells released elementary bodies at the end of the chlamydial replication cycle, but did not reflect the loss of adhesion and of polarized cell shape of all cells within the epithelium as observed in our study.

Hvid, et al. (2007) reported destruction within the epithelial layer of human FT tissue after infection with *C. trachomatis* 5 days p.i.. Mainly ciliated cells lost association with the epithelial layer and rupture occurred in some secretory cells as observed by scanning electron microscopy of the apical side. In cross-sections the epithelial layer seemed partially flattened; however, the extent and possible change of adhesion proteins can not be evaluated from these data.

Adhesion between neighbouring cells is mediated among others by adherens junctions, protein complexes that span the extracellular space between cells and are connected to the intracellular actin cytoskeleton. In polarized epithelial cells, as they are present in intact mucosa, these junctions completely encircle the cells to form the lateral adhesion belt and are important for maintaining tissue integrity. Transmembrane cadherin proteins connect the cells by homophilic interactions depending on Ca^{2+} (Meng and Takeichi, 2009; Chitaev and Troyanovsky, 1998). The redistribution of E-cadherin to the apical side of epithelial cells in infected FT tissue observed in the present study suggested a loss of cell adhesion and apico-basal polarity triggered by the infection. EGTA, a chelating agent binding Ca^{2+} and thereby inhibiting E-cadherin-dependent cell adhesion (Chitaev and Troyanovsky, 1998), was used to test this hypothesis. In EGTA treated FT tissue, E-cadherin was observed also at the apical side of cells, and the epithelial cells lost cell adhesion and columnar shape. This observation was consistent with the changes observed in chlamydial infection and thus indicated loss of epithelial cell adhesion in infected tissue in this study.

Epithelial barrier function is maintained by tight junctions. These protein complexes separate apical from baso-lateral membranes, thus being characteristic of polarized cells,

and contain the integral membrane protein occludin (Furuse, 2010; Furuse, et al., 1993). In our study, delocalization of E-cadherin to apical membrane compartments not only suggested loss of cadherin-mediated cell adhesion, but also loss of apico-basal cell polarity. For confirmation, occludin localization was analyzed in FT tissue infected with *C. trachomatis* in our group (Kessler, et al., submitted). Subapical occludin as detected in non-infected tissues was lost after infection and delocalized. These results further support the findings presented here.

The loss of adhesion and polarity observed in this study is apparently directly regulated by the pathogen. Apico-basal polarity and structure is crucial for epithelial function (Gibson and Perrimon, 2003). Their disturbance in *C. trachomatis* infection may contribute to pathology. Interestingly, similar phenotypes were reported from infections with other bacterial pathogens. A loss of E-cadherin and cytokeratin-18 was observed in intestinal epithelial cells after infection with *Escherichia coli* (Cane, et al., 2010). Guttman, et al. (2006) reported the disruption of tight junctions characterized by delocalization of several claudin-proteins in an *in vivo* mouse study of enterohaemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) infection. *Helicobacter pylori*, a stomach pathogen, secretes effector proteins into epithelial cells by a type IV secretion system. These effectors induce loss of cell adhesion in gastric epithelial cell culture. The mechanisms were shown to include disruption of adherens junctions and E-cadherin cleavage (Weydig, et al., 2007; Suzuki, et al., 2005). *Campylobacter jejuni* intestinal infection disrupted claudin-4 localization at tight junctions and led to loss of epithelial barrier function in newly hatched chicken (Lamb-Rosteski, et al., 2008). Additionally, there are viruses known to interfere with cell-cell contacts as well. The Ebola virus envelope glycoprotein was described to induce loss of cell adhesion with cell rounding and detachment in cell lines and primary cells. Levels of cell death did not increase and cells were able to re-adhere and divide after transient glycoprotein expression (Simmons, et al., 2002).

4.2.4 Apoptosis is induced during infection with *C. trachomatis* serovar D but not serovar L2

The *Chlamydia trachomatis* serovar D is associated with urogenital tract infections. Tissue culture infection by *C.tr*achomatis was mainly analyzed using this strain in the present study. A widely used laboratory strain, serovar L2, was incorporated into this study to determine possible differences between the strains. In cell culture experiments, *Chlamydia trachomatis* has been widely described to inhibit apoptosis. Interestingly, we showed in this study that infection with *C. trachomatis* serovar D induced apoptosis. Apoptosis was not restricted to infected cells. Phenotypic changes in non-infected cells

could be significant for and contribute to the understanding of *Chlamydia*-induced pathology, as they suggest the existence of a paracrine signalling during acute infection and change in epithelial homeostasis. The induction of apoptosis was observed in tissue explants as well as in cell culture. The serovar L2 did not induce apoptosis, as expected from literature.

So far, bacteria of the family *Chlamydiaceae* have been extensively described in literature to confer apoptosis resistance on their host cells. Reports include the human pathogenic species *Chlamydophila pneumoniae* and *Chlamydia trachomatis* (Böhme, et al., 2010; Greene, et al., 2004; Rajalingam, et al., 2001; Fan, et al., 1998). Inhibition of apoptosis is known from literature among others for the *C. trachomatis* serovar L2 (Böhme, et al., 2010; Rajalingam, et al., 2006; Pirbhai, et al., 2006; Ying, et al., 2005; Fischer, et al., 2004; Fan, et al., 1998).

However, there are few reports about induction of apoptosis following infection with chlamydiae in a caspase-independent manner (Perfettini, et al., 2002; Ojcius, et al., 1998) or only recently described via caspase-8 (Vats, et al., 2010). These results are discussed controversially in the literature. Chlamydiae seem to possess the ability to both inhibit and induce apoptosis, as was already reviewed by Häcker, et al. (2006) and Byrne and Ojcius (2004). In contrast, some authors say the observed cell death was different from apoptosis due to lack of caspase activation in cells infected with *C. trachomatis* serovar L2 and the cleavage of host proteins by the chlamydial protease-like activity factor (CPAF) (Paschen, et al., 2008; Ying, et al., 2006).

Attempting to clarify the controversy about reported inhibition and induction of apoptotic cell death by chlamydiae, Greene, et al. (2004) used 17 different chlamydial strains (including 14 *C. trachomatis* serovars) in comparison to analyze chlamydial effects on host cell apoptosis. The authors stated that no biologically significant apoptosis was observed during infection with any of the serovars, and that an anti- but not pro-apoptotic activity predominated in cultures infected with chlamydiae. Nevertheless, low levels of apoptotic Hela cells (< 5 %) were detected at early time points (24h, 30h) in mostly non-infected cells in infections with many serovars. Additionally, two serovars caused apoptosis also in infected cells 24h p.i., including *C. trachomatis* serovar D. At this time point no apoptosis at all was detected in completely non-infected cultures. However, when apoptosis was induced with staurosporin 4h prior to the end of experiments, all serovars conferred apoptosis resistance on infected cells compared to non-infected cells in the same culture. This included serovar D.

The observed differences might be due to different infection conditions. Greene, et al. (2004) did not specify the subtype of Hela cells, and used MOI 1 for infection with serovar D and infection medium containing 10 % FCS. We used higher MOI (MOI of 3) in cell

culture and only 5 % FCS resulting in better infections. However, resistance to an external apoptotic stimulus was not tested in this study. Likewise, Zhong, et al. (2006) included *C. trachomatis* serovars D and L2 in their studies of chlamydial infection in Hela cells using 10 % FCS infection medium and an MOI of only 0.2. Both serovars L2 and D inhibited apoptosis when it was induced by treatment with staurosporin. Only infected cells were protected. Again, experimental differences might account for the varying observations.

Indeed, chlamydiae seem to possess apoptosis inhibiting as well as inducing capacities. As active caspase-3 was detected in the present study, the observed form of cell death is considered to be apoptosis. Inhibition of apoptosis might be only exerted in the presence of pro-apoptotic stimuli and might be of greater importance in long-term infections.

Other obligate intracellular bacteria were described to inhibit apoptosis. In case of the Gram-negative bacterium *Rickettsia rickettsii* activation of the transcription factor NFκB, which leads to expression of anti-apoptotic genes, is necessary for host cell survival (Clifton, et al., 1998). The facultative intracellular bacterium *Shigella flexneri* as well as enteropathogenic *Escherichia coli* (EPEC) were both described to inhibit apoptosis by a type III secretion system secreted factor (Hemrajani, et al., 2010; Faherty and Maurelli, 2009).

Some pathogens induce apoptosis in infected cells by a bacterial toxin. *Listeria monocytogenes* can invade and move inside host cells. It produces listeriolysin, which triggers apoptosis in dendritic cells and might inhibit immune responses (Guzmán, et al., 1996). Shiga toxin expressed by *Shigella dysenteriae* can induce apoptosis in a variety of cells, including epithelial and lymphoid cells (Tresh, 2010). Induction of apoptosis was reported as well for the human stomach pathogen *Helicobacter pylori*, triggering apoptosis in gastric epithelial cells (Chu, et al., 2003).

Thus, both apoptosis induction and inhibition are widely distributed among pathogenic bacteria. These mechanisms may lead to death of immune cells or access to further cell layers in case of apoptosis, or a prolonged infection inside cells when apoptosis is inhibited.

4.3 Perspective

In this study, reversible immortalization was applied to human primary cells of the umbilical vein and the fallopian tube (FT). While the umbilical vein cells expressed an endothelial marker protein, immortalized FT cells expressed markers that are found in stem cells. Future experiments could further resolve the cell type of the generated immortalized FT cell lines, by investigating the occurrence of additional stem cell markers. The mesenchymal stem cell markers CD13 and CD73 (an aminopeptidase and a nucleotidase, respectively) were described for human FT stem cells, as well as the adhesion markers CD29 (integrin β1) and CD90 (cell surface glycoprotein Thy-1) (Jazedje, et al., 2009).

In addition, it is interesting to see whether the immortalized cells retain anchorage-dependent growth or were transformed and tumorigenic. Invasiveness of cells can be tested *in vitro* with an invasion assay. Cells are grown on a matrix consisting of basement membrane components and observed for cell invasion into the matrix. Alternatively, cells are grown on a coated porous membrane and the percentage of cells is determined that migrated through to the other side of the membrane. *In vivo* experiments are described in literature, too, to analyze tumorigenicity. The tumor forming potential of cells can be tested in animal experiments by injection of cells into nude mice and subsequent examination of tumors.

This study also showed that the *ex vivo* tissue culture of human FT is a valid and reliable model system. It includes higher complexity compared to tissue culture models and comprises cells in their natural tissue context. In this system it is possible to analyze cellular events as well as the interplay between cells or between pathogen and host cell on a subcellular level and at the same time in an *in vivo*-like situation.

Infection with *Chlamydia trachomatis* revealed damage of the epithelial layer in the FT tissue, during which epithelial cells lost polarity and adhesion. Further analysis is required to determine the molecular events following the disruption of the epithelial layer. The adherens protein E-cadherin was shown in this study to be delocalized after chlamydial infection. In addition to the structural function of full-length cadherins in adherens junctions, their extra- and intracellular cleavage fragments fulfil biological functions, too. It is known that the cytoplasmic fragment of E-cadherin is involved in intracellular signalling processes and localizes to the nucleus, where it can bind DNA. Additionally, E-cadherin cleavage increases the levels of soluble α- and β-catenin in the cytoplasm (Ferber, et al., 2008; Marambaud, et al., 2002). Thus, E-cadherin structural changes and signalling as well as other proteins important for cell polarization might be influenced by or involved in the epithelial destruction.

Apoptosis inhibition has been widely described as a characteristic observed in chlamydial infections. In this study, differential behaviour was shown for a chlamydial strain that induced apoptosis. Molecular analysis is necessary to determine how components of the apoptosis pathway are differently influenced by chlamydial strains. Chlamydiae can prevent the permeabilization of mitochondria, which is a pro-apoptotic step, by interfering with upstream factors, e.g. inhibiting BH3-only proteins and stabilizing Bcl-2 family proteins. Chlamydiae can also prevent the activation of caspase-3, a key effector of apoptosis, by increasing the levels of inhibitory IAPs (Sharma and Rudel, 2009). An apoptosis-inducing chlamydial strain might interact with these factors in another way.

There are restrictions of the tissue culture model system regarding possible manipulations. In cell culture systems, gene silencing has evolved as a useful tool to inhibit single factors and investigate their influence on cellular mechanisms, including pathogen-host-interactions. Today it is still not possible to generate targeted mutants of chlamydiae. Therefore, the host cell is the possible point of manipulations. However, cells in tissues are much more resistant to transfection of silencing constructs, such as siRNAs, due to the stable and complex integration of cells in tissues. Additionally, only the exterior cell layers of tissues will most likely be reached by silencing constructs. Future experiments will determine the possibility and efficiency of delivering silencing constructs into human FT tissue, e.g. by viral transfer with lenti- or adenoviruses or by transfection reagents designed for *in vivo* animal studies. Transfer of siRNA is investigated for other tissue (Forbes, et al., 2009). Gene silencing in tissue explants would provide a model system combining the qualities of *in vivo*-like experiments and cell culture usability.

In conclusion, this is the first study investigating chlamydial infection processes in an *ex vivo* tissue model with detailed analysis on a subcellular level. In addition, this study presents immortalization and characterization of human FT cells and the strategy of reversible immortalization. Stable *ex vivo* tissue culture and successful reversible immortalization are useful models to provide insights into the infection processes of *Chlamydia* and of other pathogens, e.g. *Neisseria*. Thus they can contribute to the broader understanding of bacterial infections.

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List of abbreviations

Bmi1	B lymphoma Moloney Murine Leukemia Virus insertion region 1
CMV	Cytomegalovirus
CPAF	chlamydial protease-like activity factor
dic	dicentric chromosomes
EB	elementary body
EGTA	ethylene glycol tetraacetic acid
EM	electron microscopy
FCS	fetal calf serum
FFPE	formalin-fixed paraffin embedded (tissue)
FT	fallopian tube(s)
Gfp	green fluorescent protein
h	hour(s)
HPV	human papillomavirus
HSV-1	Herpes Simplex Virus type 1
hTERT	human telomerase reverse transcriptase
HUVEC	human umbilical vein endothelial cells
IAPs	inhibitor of apoptosis proteins
LV particles	lentivector particles
mar	marker chromosomes
min	minute(s)
MOI	multiplicity of infection
Pen	Penicillin
p.i.	post infection
PID	Pelvic inflammatory disease
RB	reticulate body
ring	ring chromosomes
RT	room temperature
RT-PCR	real time reverse transcriptase-PCR
STD	sexually transmitted disease(s)
Strep	Streptomycin
SV40T	Simian Virus 40 large tumor antigen
TK	thymidine kinase
TTSS	type III secretion system
VSV-G	Vesicular Stomatitis Virus Glycoprotein

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Publications

***Chlamydia trachomatis* disturbs epithelial tissue homeostasis in the human Fallopian tube via paracrine Wnt signalling**

Mirjana Kessler, Julia Zielecki, Oliver Thieck, Hans-Joachim Mollenkopf, Christina Fotopoulou and Thomas F. Meyer

(submitted)

Tarp regulates early *Chlamydia*-induced host cell survival through interactions with the human adaptor protein SHC1

Adrian Mehlitz, Sebastian Banhart, André P. Mäurer, Alexis Kaushansky, Andrew G. Gordus, Julia Zielecki, Gavin MacBeath and Thomas F. Meyer.

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Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Berlin, den

Julia Zielecki